



Peer Reviewed

Title:

Pharmacological analysis of the cholinergic and serotonergic signaling systems in *Caenorhabditis elegans*

Author:

[Cregg, James F.](#)

Acceptance Date:

2010

Series:

[UC San Diego Electronic Theses and Dissertations](#)

Degree:

Ph. D., [UC San Diego](#)

Permalink:

<http://escholarship.org/uc/item/5xh8h237>

Local Identifier:

b7021783

Abstract:

The nervous system is designed to allow an organism to react to its changing environment in a coordinated manner. The simple nervous system of *Caenorhabditis elegans* has been a model for molecular studies in neurobiology for over four decades. This organism has contributed to our understanding of the molecules of neurotransmission and behavioral genetics. Here we seek to further characterize the molecules and simple circuits which lead to behaviors with hopes to generalize our knowledge from worms to higher organisms. Worms' sinusoidal locomotion is based on the coordinated contraction of body wall muscles (BWM) mediated by cholinergic signaling. Cholinergic signaling at the neuromuscular junction (NMJ) is mediated by the nicotinic acetylcholine receptors (nAChRs), this is also the site of psychotropic activity of the addictive drug nicotine. Our ultimate goal of this research was to elucidate the molecular mechanisms involved in cholinergic signaling to further understand nicotine addiction. We initiated a screen to find effectors of the cholinergic signaling system to further our understanding of the molecular elements involved in neurotransmission. We over-stimulated the BWMs with a cholinergic agonist, nicotine, causing paralysis. Loss of molecules involved with cholinergic neurotransmission ameliorates the nicotine-induced paralysis. Utilizing a RNA interference (RNAi) library to systematically knock down each predicted gene products on chromosome one, we attempt to find new molecules that play a role in cholinergic signaling. This screen produced several hits, here we analyze one, agef-1, an ADP-ribosylation factor's guanine nucleotide exchange factor (Arf-GEF). Advancements in cell physiologic techniques in *C. elegans* have been revolutionary in extending the capabilities of researchers of this organism. Using calcium imaging, we examine the activity of specific cells in relation to behaviors. A small circuit of neurons and muscles control the egg-laying behavior. This simple design belies its molecular complexity. Egg-laying is controlled by at least



two neurotransmitters/neuromodulators, acetylcholine (ACh) and serotonin (5-HT)--as well as neuropeptides. We use calcium imaging to describe the response of the egg-laying circuit to pharmacological agents in order to understand the molecular signaling networks underpinning its control

Copyright Information:

All rights reserved unless otherwise indicated. Contact the author or original publisher for any necessary permissions. eScholarship is not the copyright owner for deposited works. Learn more at http://www.escholarship.org/help_copyright.html#reuse



eScholarship
University of California

eScholarship provides open access, scholarly publishing services to the University of California and delivers a dynamic research platform to scholars worldwide.

UNIVERSITY OF CALIFORNIA, SAN DIEGO

Pharmacological Analysis of the Cholinergic and Serotonergic Signaling Systems in
Caenorhabditis Elegans

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

in

Biology

by

James F. Cregg

Committee in charge:

Professor Darwin Berg, Chair
Professor William Schafer, Co-Chair
Professor Anirvan Ghosh
Professor Amy Pasquinelli
Professor Palmer Taylor

2010

Copyright

James F. Cregg, 2010

All rights reserved.

The dissertation of James F. Cregg is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

Co-Chair

University of California, San Diego

2010

DEDICATION

To my parents, Dr. James M. Cregg and Mrs. Jeannie Cregg, whose unwavering support since before I can remember has allowed me to become a reasonably productive human being.

TABLE OF CONTENTS

Signature Page	iii
Dedication	iv
Table of Contents	v
List of Figures	ix
List of Tables	xi
Abbreviations in Use	xii
Acknowledgements	xiii
Vita	xvii
Abstract of the Dissertation	xix
Chapter I Introduction	1
<i>C. elegans</i> as a Model Nervous System	3
Molecular Signaling in <i>C. elegans</i>	4
Cholinergic Signaling	5
Serotonergic Signaling	7
G-protein Signaling	8
Behavioral Analysis and Screens	11
Locomotion	15
Egg-laying	17
Neurophysiology in <i>C. elegans</i>	24
Electrophysiology.....	25

Calcium Imaging	26
Conclusion	28
Chapter II Molecular Mechanisms of Serotonergic and Cholinergic Control of	
Egg-laying in <i>C. elegans</i>	30
Abstract	31
Introduction	32
Results	35
Mutation of 5-HT Receptors has no Effect on ACh Stimulation of the VMs	35
Mutation of all 5-HT Receptors Abolishes 5-HT Induced Activity in the VMs,	
but Raises Basal Activity	38
Basal Activity is not Mediated by an Individual 5-HT Receptor	40
Mutation in Any 5-HT Receptor Reduces Sensitivity to 5-HT in VMs	41
Each 5-HT Receptor Confers a Unique VM Response to 5-HT	42
5-HT Inhibits VM Activity through <i>mod-1</i> and <i>ser-4</i>	43
<i>ser-4</i> is Expressed in the VMs	44
VM Expressed SER-4 Mediates 5-HT Inhibition of the VM	44
Adaptation to Serotonin is Mediated by SER-4 in the VM	47
Single 5-HT Receptors that in Isolation Adapt to 5-HT, Synergistically	
Combine to not Adapt to 5-HT	49
Discussion	50
ACh Excites VM Directly Through nAChRs	50
SER-4 Inhibition of VM Activity and Egg-laying	52

Vulval Muscle Expressed SER-4 is Required for 5-HT Adaptation	54
Materials & Methods	56
Chapter III High-throughput RNAi Screen for Molecules Involved in Cholinergic	
Transmission	76
Abstract	77
Introduction	78
Results	83
RNAi Depletion Confers Resistance to Nicotine-Induced Paralysis	83
Maximizing the Effect of RNAi	85
Titration of Nicotine to Maximize Valid Hits	87
Pilot Screen of Chromosome One	89
Re-screening Putative Hits	91
Resistance to Non-Cholinergic NT Receptor Agonists	92
Nicotine Resistance on Plates	94
Testing the Mutants for Nicotine and Levamisole Resistance	95
Discussion	96
Genes Identified in the Screen of Chromosome One	96
Mutant Phenotypes	97
Materials & Methods	101
Chapter IV Characterization of the Nicotine Resistance Factor <i>agef-1</i>	115
Abstract.....	116
Introduction.....	116

Results	120
Identification of <i>agef-1</i> in RNAi Screens	120
Description of <i>agef-1</i> and AGEF-1	121
<i>agef-1</i> Expression	122
Cholinergic Agonist Resistance	123
Aldicarb and Muscimol Resistance Testing	123
Brefeldin A, Arf-GEF inhibition	124
Effect of <i>agef-1</i> (RNAi) on the Expression Level of an nAChR Subunit	126
Dominant Negative to Eliminate AGEF-1 Activity in BWM	128
Discussion.....	132
Materials & Methods	146
Appendix I Putative Nicotine Resistance Factors Identified in Screening of	
Chromosome One	146
Appendix II Getting the Most Out of an RNAi Screen	154
References	158

LIST OF FIGURES

Chapter II

Figure 2.1	Egg-laying System Diagram	65
Figure 2.2	VM Stimulation by ACh	67
Figure 2.3	Serotonin Strongly Stimulates VM Activity	68
Figure 2.4	5-HT Activity Induced in Single 5-HT Mutants	69
Figure 2.5	Dosage Curve for Quadruple Mutants	70
Figure 2.6	SER-4 and MOD-1 Inhibit VM Activity in Response to Serotonin	71
Figure 2.7	Expression of the Integrated Pser-4::GFP, <i>1jIs3</i>	73
Figure 2.8	SER-4 Expressed in the VMs is Required for Adaptation to Serotonin..	74

Chapter III

Figure 3.1	RNAi of Cholinergic Signaling Genes Confers Nicotine Resistance	103
Figure 3.2	Induction of RNAi by Feeding Versus Soaking	105
Figure 3.3	Two-generation Versus Post-embryonic Induction	107
Figure 3.4	Determination of Nicotine Dosage	108
Figure 3.5	Post-embryonic Induction of RNAi and Nicotine Resistance Assay	109
Figure 3.6	Putative Functions of the Nicotine Resistance Factors	110
Figure 3.7	Putative Nicotine Resistance Factors not Resistant to Muscimol	114

Chapter IV

Figure 4.1	<i>agef-1</i> Gene and Protein, tm1693 Deletion and E607K Mutation	136
------------	--	-----

Figure 4.2	<i>agef-1</i> (RNAi) Confers Resistance to Cholinergic Agonists	137
Figure 4.3	<i>agef-1</i> (RNAi) Confers Aldicarb but not Muscimol Resistance	139
Figure 4.4	Heterozygous <i>agef-1</i> Mutation [<i>agef-1 (tm1693/+)</i>] Confers Cholinergic Agonist Resistance	141
Figure 4.5	Putative Dominant Negative AGEF-1 (E607K) does not Confer Resistance to Nicotine	143
Figure 4.6	Quantification of Surface Expression of an nAChR Subunit in <i>agef-1</i> (RNAi) and Heterozygous <i>agef-1</i> Mutants.....	145

LIST OF TABLES

Chapter III

Table 3.1	Putative Functions of Genes Mediating Nicotine Resistance	113
-----------	---	-----

Appendix I

Table A.1.1	Putative Nicotine Resistance Factors Identified in Screening of Chromosome One	147
-------------	---	-----

ABBREVIATIONS IN USE

Abbreviation	Definition
5-HT	5-hydroxytryptamine (serotonin)
GABA	γ -aminobutyric acid
ACh	acetylcholine
AChE	acetylcholine esterase
ADP	adenosine diphosphate
Arf	ADP-ribosylation factors
bp	basepair(s)
BWM	body wall muscle
CFP	cyan fluorescent protein
cAMP	cyclic adenosine monophosphate
dsRNA	double-stranded RNA
egl-c	egg-laying constitutive
egl or egl-d	egg-laying defective
EM	electromicroscopy
ER	endoplasmic reticulum
FRET	fluorescence resonance energy transfer
GPCR	G-protein-coupled receptor
GAP	GTPase-activating G-proteins
GEF	guanine nucleotide exchange factor
G protein	guanine nucleotide-binding protein
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HSNs	hermaphrodite specific neurons
kb	kilobasepairs
lev	levamisole (cholinergic agonist)
NGM	nematode growth medium
NMJ	neuromuscular junction
NT	neurotransmitter
nAChR	nicotinic acetylcholine receptor
PDMS	Polydimethylsiloxane
RNAi	RNA interference
RT	room temperature
SEM	standard error of the mean
VC(1-6)	ventral cord type-C neurons
VCs	ventral cord type-C neurons 4 and 5
VMs	vulval muscles (class 1 & 2)
VM1s	vulval muscles class 1
VM2s	vulval muscles class 2
N2	wild-type (<i>C.elegans</i> Bristol strain)
YFP	yellow fluorescent protein

ACKNOWLEDGEMENTS

Chapter two is being prepared as a manuscript for publication. We are grateful to Dr. Amy Pasquinelli and Dr. Bruce Bamber for allowing the author to complete portions of this work in their labs. We were generously provided with multiple receptor 5-HT mutants from by Dr. Richard Komuniecki: *ser-5; ser-4; mod-1; ser-7 ser-1* (RWK3) quintuple 5-HT mutants, *ser-5; ser-4; mod-1; ser-7* (RWK14) *quad (ser-1)* mutants, *ser-5; ser-4; mod-1; ser-1* (RWK15) *quad (ser-7)* mutants and *ser-5; ser-4; ser-7 ser-1* (RWK17) *quad (mod-1)* mutants and *ser-4; mod-1; ser-7 ser-1* (OT182) *quad (ser-5)* mutants. The custom microfluidics chamber for the high speed application of pharmacological agents was provided by our collaborator, Dr. Nikos Chronis.

My studies took me around the world and back again. From far too idyllic UCSD to the famed Laboratory of Molecular Biology in Cambridge, England, to Toledo, Ohio, in the middle of winter and, finally, to San Francisco. While this situation was less than optimal for data collection and scientific productivity, I did get the opportunity to meet people from around the world and experience academic science in ways that few graduate students could possibly imagine. For these not exactly requested adventures and for exceedingly brilliant scientific mentorship I would like to thank my advisor, Dr. Bill Schafer. I could always count on his genuine excitement for science to motivate me no matter how badly the previous experiment had failed. I would also like to acknowledge Dr. Darwin Berg for his efforts above and beyond a committee member to help me succeed. (Though knowing Darwin, he would

probably say that he didn't do much at all, sometimes knowing someone is unwaveringly on your side is enough.) Also, Dr. Amy Pasquenelli, who in addition to being a valuable committee member allowed me space in her lab when I was 'denied entry' to the UK for five months. Dr. Arnivan Ghosh for his ability as a committee member to cut through it everything and get to the most interesting and critical points. And rounding out my committee, Dr. Palmer Taylor from the Pharmacology department, whose concern about my scientific future was touching and a good example for my future. I don't think that I could have assembled a better committee in terms of science, and commitment to my future success. I sincerely thank you all.

I regard, with reverence, the early years of my studies at UCSD. I don't know if I will stand shoulder to shoulder with any group of such potential. I can't imagine what my time at UCSD would be like without Eduardo Ezquenazi, one of the most gifted and generous individuals that I've ever known. The surf inspired adventures will always be my best memories, even when we didn't score the surf, it was always a good time. The person most generous of spirit that I would ever hope to meet, Jenn Stubbs, whom none of us would have survived grad school without. Val Vitkauskas for all of the great surfing memories for being the life of the party and when there was no party, just making it happen. And, of course, my longest friendship in SD, Scarlett Dalgeish, for making SD so much damn fun. Also, Matt Rardin for his patience and planning for all of our Mexico journeys. We always made it back despite my best efforts. Neal Alto for the stoke and fire on all of the early morning surf missions and for making in all look so damn easy. I would also like to thank the SD poker crew, or

more of the drink beer and insult crew with some cards somewhere around, thanks for the good time and all of your money, it almost covered the beer. Also, the beach volleyball players for all of the great times out on the beach, running between games and the ocean for a surf.

Some friends go beyond friends and become part of who you are. Ryan Hibbs is one of those rare few for me. I could write forever about the great times but I will just say thank you, I know there will be many more in the years to come. Also, Jason and Liz Lanier two of the most wonderful people you could imagine. They opened my horizons in music and thought. So different in all the things that people tell you should matter, but so much the same in all of the things that really do. (Sorry, Jason, I borrowed a bit of the Texan accent in the UK just to be sure there was no confusion as to me *not* being English.)

I must thank all of my UCSD lab mates for training me, and giving me the room to grow. I would like to mention Alex Gottschalk for all the help he gave me starting off the nicotine projects. I would especially like to mention Katie Kindt and Kathleen Quast, for keeping the lab and microscopes operational and for friendship in the lab and out of it. As well as Callie and Katya for keeping everyone in good spirits and as well grounded as we could be—which really wasn't that well grounded, but I really couldn't imagine what sort of lunacy would have run rampant in our lab without them.

The greatest thing about a nomadic existence is, without a doubt, the people you meet along the way. My experiences in Cambridge allowed me the opportunity to

meet many people that I would never have met in the US. My house mates in Cambridge, Simone, Tuffa and Rachael, were my family while in the UK. My lab mates in Cambridge, whom I spend long hours and had great times with, especially Tadas and Marios, who kept late hours and kept me motivated in the dark. After the UK was a stop in Toledo, Ohio, in the middle of winter, not my top choice of places to finish my thesis work, but there I found Bruce and Kathleen Bamber, who took me into their home and made me welcome. Their warmth and kindness—and many home cooked meals—turned what might have been terrible drudgery into an enjoyable one. My Uncle Mike was also instrumental in keeping me sane while in the cold. Having good family near makes anything better.

One of the hardest things about a wandering life is holding on to yourself in the chaos that, ultimately, becomes your accepted, normal life. It is easy to lose sight of yourself in such situations. These people are those that ground you and define who you are—or, sometimes, who you hope to become. For that I reserve special thanks in my heart for my long-suffering girlfriend, Lindsay Hall, whose calls were a lifeline from home. Whose visits and support sustained me and gave me hope that were reasons to fight for what you love. And for the future ahead of us, as we make our way in a new city.

VITA

EDUCATION

- 2010 Ph.D., Biology, University of California, San Diego
- 2000 B.A., Biology-Chemistry Combined, Whitman College
- 1996 High school diploma, Jesuit High School, Portland, Oregon

WORK EXPERIENCE

- 2000-2002 Research Assistant, University of California, San Diego

PUBLICATIONS

CREGG, J.F., HAPIAK, V. H., *et al.* [Manuscript in preparation— Winter 2011] DISSECTING THE ROLE OF SEROTONIN AND ACETYLCHOLINE ON THE VULVAL MUSCLE MOTOR SYNAPSE IN C.ELEGANS.

Melnik-Martinez, K., Cregg, J. F., *et al.* [In Review—2010] THE CLAUDIN/STARGAZIN HOMOLOGUE CLC-3 AFFECTS CALCIUM INFLUX AT C. ELEGANS NEUROMUSCULAR SYNAPSES.

YEMINI, E., Cregg, J. F., *et al.* [Manuscript Accepted, JOURNAL OF VISUALIZED EXPERIMENTS, JULY/AUGUST 2010] IMPROVED AUTOMATED TRACKING AND ANALYSIS OF C.ELEGANS. (WWW.MRC-LMB.CAM.AC.UK/WORMTRACKER/)

TEACHING

BIPN 145 Neurobiology Lab, Teaching Assistant
Lecturer Karen Ocorr, University of California, San Diego Fall 2003

BIMM 101 Recombinant DNA Techniques, Teaching Assistant
Dr. Mandy Bulter, University of California, San Diego, Winter 2005

FIELDS OF STUDY

Major Field: Biology

Studies in Molecular and Genetic Neurobiology Biology

Professor William Schafer

University of California, San Diego

Laboratory of Molecular Biology, Medical Research Council, Cambridge, UK

Studies in Genetics and Molecular Biology

Professor Scott Emr

University of California, San Diego, HHMI

ABSTRACT OF THE DISSERTATION

Pharmacological Analysis of the Cholinergic and Serotonergic Signaling Systems in
Caenorhabditis Elegans

by

James F. Cregg

Doctor of Philosophy in Biology

University of California, San Diego, 2010

Professor Darwin Berg, Chair

Professor William Schafer, Co-Chair

The nervous system is designed to allow an organism to react to its changing environment in a coordinated manner. The simple nervous system of *Caenorhabditis elegans* has been a model for molecular studies in neurobiology for over four decades. This organism has contributed to our understanding of the molecules of neurotransmission and behavioral genetics. Here we seek to further characterize the molecules and simple circuits which lead to behaviors with hopes to generalize our knowledge from worms to higher organisms.

Worms' sinusoidal locomotion is based on the coordinated contraction of body wall muscles (BWM) mediated by cholinergic signaling. Cholinergic signaling at the

neuromuscular junction (NMJ) is mediated by the nicotinic acetylcholine receptors (nAChRs), this is also the site of psychotropic activity of the addictive drug nicotine. Our ultimate goal of this research was to elucidate the molecular mechanisms involved in cholinergic signaling to further understand nicotine addiction. We initiated a screen to find effectors of the cholinergic signaling system to further our understanding of the molecular elements involved in neurotransmission. We over-stimulated the BWMs with a cholinergic agonist, nicotine, causing paralysis. Loss of molecules involved with cholinergic neurotransmission ameliorates the nicotine-induced paralysis. Utilizing a RNA interference (RNAi) library to systematically knock down each predicted gene products on chromosome one, we attempt to find new molecules that play a role in cholinergic signaling. This screen produced several hits, here we analyze one, *agef-1*, an ADP-ribosylation factor's guanine nucleotide exchange factor (Arf-GEF).

Advancements in cell physiologic techniques in *C. elegans* have been revolutionary in extending the capabilities of researchers of this organism. Using calcium imaging, we examine the activity of specific cells in relation to behaviors. A small circuit of neurons and muscles control the egg-laying behavior. This simple design belies its molecular complexity. Egg-laying is controlled by at least two neurotransmitters/neuromodulators, acetylcholine (ACh) and serotonin (5-HT)—as well as neuropeptides. We use calcium imaging to describe the response of the egg-laying circuit to pharmacological agents in order to understand the molecular signaling networks underpinning its control.

CHAPTER I

Introduction

INTRODUCTION

Behaviors are reactions to a complex and changing environment, which, at the most basic level, are based on the action of molecules within our bodies. These molecules are assembled according to the instructions contained in our DNA. That these instructions can be understood is the basic tenet of behavioral genetics. However, the study of behavioral genetics in higher organisms is hampered by the complexity of these interactions—the human brain has $\sim 10^{11}$ neurons and about 1000 times as many connections/synapses (Principles of Neural Science, 4th ed., Kandel E.R., 2000). The nematode, *Caenorhabditis elegans*, has approximately 5000 chemical synapses in the adult worm, which is about the same number of connections made by a single human hippocampal pyramidal neuron, making the task of understanding genetic control over behavior considerably less daunting (de Bono and Maricq, 2005). Its plethora of simple behaviors such as egg-laying, locomotion, chemotaxis and defecation, are highly stereotyped and easily characterized, making it a very attractive system to study behavioral genetics. Furthermore, its similarity to higher organisms in the molecules that control these behaviors makes it highly relevant to these studies. To this end, the worm has proven invaluable in the identification of new molecules related to behaviors and the signaling pathways controlling these behaviors through screens. With the advent of higher through-put techniques and directed screens utilizing RNAi, the pace at which these molecules can be identified has accelerated

greatly whereas the logistical expense has collapsed. The recent expansion of cellular physiologic techniques available to *C. elegans* researchers has opened new doors to the exploration of the behavioral responses at a circuit and network level. These techniques coupled with *C. elegans* already potent arsenal of genetic tools, such as the ability to ectopically express or knock-down a gene in almost any cell and the ability to cross mutants—available for order—all in a matter of days to weeks, make *C. elegans* an attractive organism to study the intricate molecular pathways involved in creating behaviors. Our hope is that knowledge of molecular and cellular basis of behavior in *C. elegans* can be translated to understanding more complex behaviors in humans and that therapies for pathologies, such as addictions and depression, can be aided in development by basic principles elucidated in worms.

***C. elegans* as a Model Nervous System**

C. elegans has become an important model organism for the study of neural networks. Its simple and stereotyped body plan has made it an invaluable resource for reductionist neurobiology. The hermaphrodite is composed of exactly 959 cells with 302 being neurons which have invariant cell-lineage and position which were mapped (White J.G., 1986). This group further scrutinized the cellular structure of *C. elegans* with electromicroscopy, painstakingly reassembling these serial EM sections which led to a map of the worms' cellular connections. This study generated the first neural

connectivity map and has served as the cornerstone for the continuing effort to understand neural communication at the circuit and organismal level.

Despite the relative simplicity of their nervous system, *C. elegans* utilizes many of the same neurotransmitters (NTs) and modulators as higher organisms, including, acetylcholine (ACh), γ -aminobutyric acid (GABA), glutamate, dopamine, serotonin (5-HT) and tryamine . Worms also encode several dozen neuropeptides within their genome, several of which have been demonstrated to have effects on neurons and behaviors (Ringstad and Horvitz, 2008; Waggoner et al., 2000b). *C. elegans* contains neurons that release multiple neurotransmitters/modulators, specifically, serotonin and acetylcholine (Duerr et al.). This is a possibility long theorized in mammals but only recently demonstrated (Furshpan et al.; Schutz et al.; Weihe et al., 2005). It has also been suggested that these multi-transmitter neurons might be widespread in the mammalian nervous system, further raising the already daunting complexity (Luther, 2009). The simplicity of the *C. elegans* nervous system coupled with its molecular similarity to higher organisms has given scientists a powerful reductionist platform from which to examine fundamental questions of neuroscience.

Molecular Signaling in *C. elegans*

Cholinergic Signaling

Acetylcholine is the major stimulatory NT at the *C. elegans* neuromuscular junction (NMJ), but has a number of roles in the nervous system (Brenner, 1974; Rand, 2007). Because of its role at the NJM, it figures prominently in behaviors involving muscles such as locomotion, feeding and egg-laying (Avery and Horvitz, 1989; Bany et al., 2003; Kim et al., 2001; Lewis et al., 1980). ACh's role at the NMJ has been extensively study and utilized to find novel molecules which affect cholinergic signaling. However its function in neuron to neuron communication has not been well characterized in the worm.

There are thirty-one putative ionotropic ACh receptor subunits in the worm genome (Jones and Sattelle, 2004; Putrenko et al., 2005). Twenty-seven of these are nicotinic acetylcholine receptor (nAChR) subunits; many of these have been confirmed through pharmacological studies on heterologously expressed proteins (Francis et al., 2005; Touroutine et al., 2005). Functional nAChRs are homomeric or heteromeric pentamers of these subunits and all known nAChRs are cation channels and, therefore, excitatory (Leonard and Bertrand, 2001). The other four known ACh sensitive ionotropic receptor subunits, *acc-1 – 4*, belong to a class of inhibitory ACh gated chloride channels only found in invertebrates to date (Putrenko et al., 2005). There are also three confirmed ACh sensitive metabotropic receptors, *gar-1 – 3*, which are G-coupled protein receptors — which will be discussed later (Culotti and Klein, 1983; Hwang et al., 1999; Lee et al., 1999; Lee et al., 2000).

Levamisole (*lev*), a nematode specific cholinergic agonist, is a valuable pharmacological tool for studying the excitatory cholinergic signaling in worms. It specifically targets a group of heteromeric nAChR subunits, *unc-29*, *unc-38*, *unc-63* and *lev-1*, called the levamisole-sensitive nAChRs (Lewis et al., 1987b; Lewis et al., 1980). The *lev*-sensitive nAChRs form one of two ACh sensitive receptors on the body wall muscles (BWMs), responsible for movement, and are in the egg-laying system (Richmond and Jorgensen, 1999; Trent et al., 1983). While nicotine, the canonical cholinergic agonist, targets nAChRs as well, its action is less specific, stimulating all nAChRs indiscriminately. Neither nicotine nor *lev* activates ACh sensitive chloride channels or metabotropic receptors (Putrenko et al., 2005). Both agonists are small, stable molecules that pass readily through the cuticle, the nematodes' thick outer membrane, but are slowly metabolized by the worm, if at all. ACh, the endogenous agonist, is quickly metabolized by acetylcholine esterase (AChE) and, therefore, unsuitable for studies requiring long exposure times. To study the long-term effects of ACh, an AChE inhibitor, aldicarb, is used, which increases the amount of endogenous ACh at the synapse by preventing its metabolism by AChE (Opperman and Chang, 1991; Rand and Russell, 1985). While both *lev* and nicotine work only on nAChRs, or a subset thereof, aldicarb affects all classes of ACh sensitive receptors. The worms will eventually (in 12 – 24 hrs) adapt to high levels of cholinergic agonists by down regulating the number of nAChRs in a protein kinase C dependent manner (Waggoner et al., 2000a).

Serotonergic Signaling

Serotonin (5-hydroxytryptamine [5-HT]) is a biologically active monoamine, which plays many roles in the behavior of *C. elegans* (de Bono and Maricq, 2005; Komuniecki et al., 2004). Most notably, it signals the presence of food, which in turn modulates many of the worms behaviors including the induction of egg-laying, slowing of speed—to stay on food—and stimulates the pumping of the worms' feeding organ, the pharynx (Avery and Horvitz, 1989; Ranganathan et al., 2000; Trent et al., 1983; Waggoner et al., 1998). There are eight classes of neurons that produce 5-HT, but its potential for endocrine, autocrine and synaptic signaling means that it could affect any cell in the worm (Barnes and Sharp, 1999; Sze et al., 2000).

There are five known serotonin receptors in *C. elegans*; four are G-protein coupled receptors, *ser-1*, *ser-4*, *ser-5* and *ser-7*. Three of which, *ser-1*, *ser-5* and *ser-7*, are stimulatory receptors (Hapiak et al.; Hobson et al., 2003; Tsalik et al., 2003; Xiao et al.). The final 5-HT sensitive GPCR, *ser-4*, has been shown to inhibit synaptic vesicle release as well as egg-laying—we give evidence to *ser-4*'s directly inhibitory effects here (Hobson et al., 2006; Nurrish et al., 1999; Olde and McCombie, 1997). The fifth serotonin sensitive receptor is a chloride channel, *mod-1*, which, due to its anionic specificity, is inhibitory and expressed almost exclusively in the neurons (Ranganathan et al., 2000). Collectively the 5-HT receptors are in 'almost every [neuron and muscle cell] in the worm' (Dr. Richard Komuniecki, personal communication).

The endogenous agonist, serotonin, is a stable compound but was thought not to be highly permeable to the worm's cuticle. We show here that 5-HT is more permeable to the cuticle than previously suspected, eliciting effects at 20 μ M concentrations, up to 1000 fold less than many previous assays (1.3 – 26 mM 5-HT) (Hapiak et al.; Hobson et al., 2006; Shyn et al., 2003; Trent et al.). Mammalian serotonin receptor research has been greatly aided by subtype specific drugs to deduce the contributions of each subtype of receptor (of which there are ~15) (Barnes and Sharp, 1999). Despite sequence homology between many *C. elegans* and mammalian 5-HT receptors, their pharmacological profiles and binding kinetics are quite divergent (Hapiak et al.; Hobson et al., 2006; Komuniecki et al.). However, the powerful genetic tools of *C. elegans* mitigate much of this problem, as sub-type specific drugs can be mimicked by mutations in all but one receptor or any combination thereof. Worms will also adapt to high levels of serotonin through an unknown, protein kinase A dependent mechanism which suggests desensitization of the receptors (Schafer et al., 1996; Waggoner et al., 1998). This adaptation takes 4 hours to return to basal levels of egg-laying—5-HT induces egg-laying—however, continued exposure to 5-HT causes eggs to be retained, to the point where they hatch within the adult (Carnell et al., 2005; Schafer and Kenyon, 1995).

G-protein Signaling

As previously mentioned, three ACh and four of the five serotonin receptors are a class of transmembrane metabotropic receptors called G-protein coupled

receptors (GPCRs). These receptors transduce an external signal, in this case ACh or 5-HT, through the cell membrane to activate a group of heteromeric G-proteins bound to the GPCR to initiate intracellular signaling (Bastiani and Mendel, 2006). This signaling may activate ion channels, stimulate the release of neurotransmitters, increase cyclic adenosine monophosphate (cAMP) in the cell or inhibit other pathways, among other functions in the cell (Nurrish et al., 1999). The heteromeric G-protein complex consists of three distinct protein classes, $G\alpha$, $G\beta$ and $G\gamma$, which separate after stimulation of the receptor into two signaling subunits, the $G\alpha$ monomer and the $G\beta\gamma$ heterodimer, either or both can be intracellular signaling molecules. In the classical heteromeric G-protein cycle the receptor is activated which stimulates the exchange of the guanine nucleotide guanosine diphosphate (GDP) bound to the $G\alpha$ subunit for the higher energy guanosine triphosphate (GTP). This causes the disassociation of the G-proteins from the receptor and the $G\alpha$ subunit from the $G\beta\gamma$ dimer allowing them to interact with their downstream effectors. Hydrolysis of the GTP bound to the $G\alpha$ protein causes the $G\alpha$ subunit to re-associate with the $G\beta\gamma$ subunits, terminating signaling.

There are four major classes of $G\alpha$ proteins in mammals determined by sequence homology, $G\alpha_s$, $G\alpha_q$, $G\alpha_{i/o}$ and $G\alpha_{12}$, *C. elegans* has a single representative of each of these classes, *gsa-1* ($G\alpha_s$), *egl-30* ($G\alpha_q$), *goa-1* ($G\alpha_{i/o}$) and *gpa-12* ($G\alpha_{12}$) (Bastiani and Mendel, 2006; Jansen et al., 1999). These are widely expressed throughout the worm. Generally stimulatory *egl-30* promotes the release of synaptic vesicles and contraction of muscles, whereas, *goa-1* antagonizes *egl-30* activity,

among other inhibitory activities (Hajdu-Cronin et al.; Miller et al., 1999). Activation of GSA-1 has been shown to increase cAMP levels and modulates the *egl-30* activation. The other seventeen G α subunits (*gpa-1 – 11*, *gpa-13 – 17* and *ord-3*) lack homology to classify, but are mainly restricted to amphid and other sensory neurons, with the notable exceptions of *gpa-7* and *gpa-16*, which are also widely expressed. Each GPCR is paired with a specific G α protein, but each G α protein may be activated by multiple GPCRs, even in the same cell—such as *goa-1* activation by *egl-6*, *gar-2* and *egl-47*, all in the hermaphrodite specific neurons (Bany et al., 2003; Ringstad and Horvitz, 2008). Worms contain two G β proteins *gpb-1* and *gpb-2*, and two G γ proteins, *gpc-1* and *gpc-2*, which are all widely expressed (Bastiani and Mendel, 2006).

The classical model of heteromeric G-protein signaling—as previously described—is only the tip of the iceberg of G-protein signaling. At almost any stage of the pathway, accessory proteins are capable of modifying the action of the pathway. These function mainly by altering guanine nucleotide bound to the G α protein, transitioning between the active, GTP-bound monomeric signaling state, and the inactive, GDP-bound heterotrimeric state. Guanine-nucleotide exchange factors (GEFs) catalyze the exchange of the GDP for GTP, activating the G-protein signaling complex (Donaldson and Jackson). In the classical GPCR model, the GPCR itself acts as a GEF to catalyze the activation of the complex—though GPCRs are rarely considered or classified this way. It should be noted, that the role of G-proteins and GEFs as signal modifying molecules within the cell extends beyond the G α class of

G-proteins discussed here. Conversely, GTPase-activating proteins (GAPs) catalyze the rate of GTP hydrolysis, acting as negative regulators to the G-protein signaling pathway. Furthermore, G-proteins have been shown to have receptor independent functions, especially $G\alpha_o$, some with lethal consequences (Bastiani and Mendel, 2006).

There is a second class of G-proteins in the cell not associated with GPCRs, belonging to the ras superfamily, which is divided into a number of families, including Rho (cytoskeletal dynamics), Rab (membrane trafficking) and Arf (vesicular transport) (Donaldson and Jackson). These G-proteins are similar to $G\alpha$ proteins in the heteromeric G-protein model in both structure and function, binding GTP to activate the signaling cascade and hydrolyzing it to GDP to terminate signaling. These ras family G-proteins have distinct sets of supporting G-proteins also termed GEFs and GAPs which are similar in function and structure to their counterparts in the heteromeric, but interact with only their subfamily's members (e.g. RhoGEFs act on Rho class G-proteins and Arf-GEFs act on Arfs) (Li et al., 2004).

Behavioral Analysis and Screens

Behavioral analysis and screening were original purposes for which *C. elegans* was selected as a model organism, and are still the basis for some of its most important contributions to science. *C. elegans*' small size, simple, well characterized behaviors and molecular similarity to higher organisms have made the worms an excellent

vehicle for gene discovery. The diminutive size of *C. elegans* is advantageous for large-scale screening, allowing researchers to quickly screen for behavioral or morphological abnormalities with minimal logistical expense. Reverse genetic, mutagenesis screens, search through hundreds of thousands of genomes for abnormalities caused by random, low frequency mutations. The recent discovery of RNA mediated interference (RNAi) and its development as tool for screening has dramatically changed the search for novel molecules in *C. elegans*. RNAi is the degradation of specific mRNA by introducing corresponding double stranded RNA (dsRNA) creating a depletion of the gene product. The ease of initiating RNAi and the knowledge of the target allows for high throughput forward genetic screens on the genome wide scale, which was previously restricted to reverse genetic efforts (Boutros and Ahringer, 2008). RNAi based screens allow greater flexibility in the design of the screen, as targets are known RNAi makes large candidate based screens possible. These more restricted candidate based approaches can allow for more in depth screening methods such as the axon guidance and axon regeneration screens which would have been very difficult with whole genome sized screen or random, low frequency mutagenesis screens (Yanik et al., 2004).

Locomotion and egg-laying, two of the simplest, most characterized behaviors, have been the basis of screens with far reaching implications. The first behavioral screen undertaken in *C. elegans* identified mutants with impaired locomotion—uncoordinated: unc class mutants—by Brenner and colleagues (Brenner, 1974). Locomotion requires a delicate balancing of signals for efficient, coordinated

motion; even slight defects in these molecules result in visibly uncoordinated locomotion. Many of these ‘unc’ mutants were defective in molecules involved in neurotransmission, especially ACh signaling, since these are directly involved in locomotion (Brenner, 1974; Lewis et al., 1980). Some genes found in this study are highly conserved in molecular neurotransmission; *unc-17* and *unc-13*—corresponding to the mammalian homologues, VAcHT and munc13—are involved in neurotransmitter packaging and synaptic vesicle release, respectively, in all known nervous systems (Brose et al., 1995; Erickson et al., 1994; Rand, 1989). In the early 1980s, Dr. Jim Lewis found several of these unc mutants to be highly resistant to known ACh agonists, and cloned them to uncover that they were nAChR subunits (Lewis et al., 1987a; Lewis et al., 1980).

The effects of exogenous pharmacological agents often override the inherent controls on behaviors, probing the underlying controls and signaling of the system in isolation, raising the ‘signal’ with less ambiguity and biological noise, but sacrificing native physiologic balances. These exogenous agents often change behavior in a characteristic way—e.g. 5-HT promotes egg-laying and slows locomotion—and these effects are mediated by specific receptors and signaling pathways (Ranganathan et al., 2000; Trent et al., 1983). If the functionality of these receptors or pathways is compromised by mutation or depletion of gene product, the worms become resistant to certain effects of this drug mediated by this component of the signaling pathway. Because the natural response is often hyperstimulated, these pharmacological assays

often display phenotypes hidden or too subtle for characterization under native conditions.

Mammalian studies of receptor classes have focused on pharmacologically specific agents, such as levamisole, which activate only a certain subset of receptors allowing researchers to identify the unique functions of specific receptors within a class (Touroutine et al., 2005). Unfortunately, worm and mammalian pharmacology can be highly divergent—as with mammalian 5-HT receptor class specific compounds, having no effect on worm counterparts—denying *C. elegans* researchers compounds that have illuminated so much in mammalian studies (Komuniecki et al.). Also underscoring that pharmacology research cannot be directly translated from nematode to mammal. However, the powerful genetic techniques of *C. elegans* compensates for this loss, as genes can be easily knocked out and expressed ectopically by transgenic constructs to find their site of action, helping to define the cells and molecules involved in particular behavioral pathways. The combination of pharmacology and genetics is powerful for delineating pathways, but researchers must be careful with the pharmacologic hyperstimulation of transgenically over-expressed proteins—all transgenes are ectopic over-expressions—especially with ionotropic receptors, as they could easily produce non-physiologic effects resulting in unnatural behaviors.

While ‘uncoordinated’ is a non-specific term to define locomotion abnormalities, modern assays focus on more quantifiable aspects of locomotion such as speed, body bends, and slowing responses to food. The advent of automated

tracking systems and increased sophistication of computer aided analysis has greatly increased the range and subtlety of the quantifiable features of *C. elegans* locomotion (Cronin et al., 2006; Hardaker et al., 2001). Features such as the depth of body bend and precise measurements of relative speed would have been impossible to accurately record by eye. Automated analysis allows for correlation between multiple features, allowing researchers to link probabilities, such as the probability of a turn after a reversal, which is important to behavioral studies of decision making, learning and memory. Automated tracking machines have also aided in the characterization of the egg-laying behavior, elucidating biphasic states of egg-laying and demonstrating a significant speed increase prior to egg-laying events (Hardaker et al., 2001; Waggoner et al., 1998).

Locomotion

Locomotion is a well-characterized behavior resulting from the coordinated contraction and relaxation of the body wall muscles (BWMs) to produce a sinusoidal wave, which propels the animal (Richmond and Jorgensen, 1999). The striated BWMs extend down the ventral and dorsal sides of the worm. They are electrically coupled to their ipsidorsal or ipsiventral neighbors. The BWMs are innervated from the ventral nerve cord, the ventral BWMs are innervated by VA, VB, VC and VD class motor neurons, whereas the dorsal BWMs are innervated by AS, DA, DB and DD class motor neurons (White J.G., 1986; White et al., 1976). The BWMs are controlled by two excitatory ACh receptors and one inhibitory GABA receptor which coordinates

the muscles relaxed and contracted states to produce efficient motion (Richmond and Jorgensen, 1999).

The BWM ACh response is mediated by the heteromeric, lev sensitive nAChR complex and the alpha-7-like ACR-16 homomeric complex, as well as the inhibitory GABA receptor, UNC-49 (Bamber et al., 1999; Lewis et al., 1987a; Touroutine et al., 2005). Both nAChR complexes are sensitive to nicotine which, like levamisole, hyperstimulates both ventral and dorsal BWMs simultaneously to create a rigid paralysis. Loss of any of the lev sensitive nAChR subunits, *unc-29*, *unc-38*, *unc-63*, confers resistance to both lev and nicotine, but at the expense of coordinated locomotion (Lewis et al., 1987a). ACR-16 mediates a fast initial response to ACh but mutation confers no resistance to long term exposure to ACh agonists. Nor do *acr-16* mutants display uncoordinated movement, however, double mutants defective in the lev sensitive receptor as well, are significantly more impaired than single mutants of lev sensitive subunits (Francis et al., 2005). The GABA receptor, UNC-49, mediates the relaxation of the BWMs, pharmacologic stimulation via muscimol causes 'hyper'-relaxation of the muscles, resulting in a flaccid paralysis (Bamber et al., 2003). Contraction of one side of the muscles via ACh stimulates GABA to be released to the opposite side and vice versa to coordinate efficient movement (Richmond and Jorgensen, 1999).

Egg-laying

Egg-laying is one of the simplest and most easily quantified of *C. elegans* motor programs and, as such, is one of the most heavily studied. Egg-laying is a well characterized behavior that may offer insights into NMJ and neuron to neuron communication as well as into multi-molecular signaling networks—ACh, 5-HT, tyramine and peptides—and in small circuit analysis. The egg-laying system consists of 2 classes of neurons and 16 muscles, and, recently, four endocrine cells surrounding the vulva have been implicated in egg-laying as well, uterine-vulval cells (uv1s) (Jose et al.; Schafer, 2006; White J.G., 1986). The major neurological inputs to egg-laying muscles come from a pair of hermaphrodite specific neurons (HSNs). The HSNs are positioned bilaterally, posterior to the vulva itself while maintaining a large synapse to the egg-laying muscles as well as connections with the other class of egg-laying motor neurons, the proximal ventral type C neurons 4 and 5. The HSNs, notably, utilize both cholinergic and serotonergic signaling, they are also capable of synthesizing neuropeptides (Duerr et al., 1999; Forrester et al., 2004; Kim and Li, 2004; Nathoo et al., 2001; Schinkmann and Li, 1992). The HSNs serve as a major point of control for egg-laying, receiving inputs directly from the head, as well as sensory neurons and extrasynaptic signals (Ringstad and Horvitz, 2008; White J.G., 1986; Zhang et al., 2008). The loss of the HSNs causes the massive accumulation of eggs in the uterus, due to a slowed egg-laying rate (Trent et al., 1983). This suggests that the HSNs exert a high degree of control over egg-laying by regulating the initiation of egg-laying.

The other class of neurons that synapses onto the muscles of the egg-laying system is the ventral cord type C neurons (White J.G., 1986). These neurons, also hermaphrodite specific, consist of six cholinergic neurons positioned linearly along the ventral cord (VC1 – 6, running anterior to posterior) that innervate the vulval muscles. The most important to egg-laying are VC4 and VC5, VC4 being just anterior and VC5 just posterior to the vulva. (Note: VC4 and VC5 will be referred to as the VCs, any reference to the full set of ventral cord type C neurons will be denoted as VC1-6.) The VCs maintain neuronal arborization (both axonal and dendritic) with the HSNs and are the only neurons of VC1-6 to exclusively innervate the egg-laying muscles (White J.G., 1986). The role of the VCs is complex; ablation studies of the VCs alone show a slight increase in egg-laying, however, when ablated in conjunction with the loss of the HSNs they further inhibit egg-laying (Waggoner et al., 1998).

The muscles of the egg-laying system consist of two classes of eight muscles each, the vulval muscles and the uterine muscles (White J.G., 1986). The vulval muscles (VMs) consist of eight smooth muscles that contract during an egg-laying event in order to open the vulva allowing the egg to be released. The VMs are sub-categorized into two sets of 4 radially oriented muscles, vulval muscle 1s (VM1s) and vulval muscle 2s (VM2s). The two sets of vulval muscles are ventrodorsally stacked, with the VM1s being more ventral, closer to the vulva. More importantly, the VM2s are the only muscles in the system innervated by the egg-laying neurons. They are electrically coupled to the VM1s via gap junctions. While having no significant synaptic inputs, the VM1s are all linked to adjacent VM1s by gap junctions as well as

their partner VM2. The innervated VM2s are required for egg-laying whereas the VM1s are completely dispensable, showing no effect on egg-laying rates (Schafer, 2006). The second class of muscles, the uterine muscles, is thin, circular smooth muscles that encircle the uterus just anterior and posterior to the vulva. Thought to play a role in embryo positioning for egg-laying, they are completely dispensable to normal egg-laying. Four uv1 endocrine cells surround the vulva and release tryamine, inhibitory to egg-laying, and, possibly, neuropeptides. These uv1s inhibit egg-laying, as ablating or silencing the uv1s cause hyperactive egg-laying (Jose et al.).

An egg-laying event is preceded by highly correlated excitation of the both classes of motor neurons, HSNs and VCs, which results in the coordinated contraction of the VMs thus opening the vulva to release an egg (Zhang et al., 2008). The egg-laying motor neurons release both ACh and 5-HT as NTs/neuromodulators (Duerr et al., 1999; Weinschenker et al., 1995). Pharmacological studies have shown that both ACh and serotonin are, paradoxically, stimulatory *and* inhibitory to this process. ACh agonists, nicotine and levamisole, stimulate egg-laying through a pathway that is HSN dependent—HSNs are the major source of 5-HT for the egg-laying system—or requires the addition of exogenous 5-HT. Curiously, ACh induced stimulation of activity in the VMs, an important step in egg-laying and down-stream of the HSNs, requires no neural input, nor exogenous 5-HT, but will not result in egg-laying (Shyn; Waggoner et al., 1998). This excitation is mediated by both the lev sensitive nAChR complex and lev insensitive nAChRs of unknown composition expressed by the VM2s as well as the VCs. Despite its requirement for egg-laying in response to cholinergic

agonists, HSN has not been shown to express any excitatory ACh receptors. In contrast to the stimulation by exogenous cholinergic agonists, inhibiting the activity of AChE—thus increasing the availability of acetylcholine—decreases the rate at which eggs are laid (Bany et al., 2003). This decrease is mediated by the ACh sensitive GPCR *gar-2*, expressed by the HSNs. GAR-2 is not sensitive to either nicotine or lev, which is why those cholinergic agonists show only ACh mediated stimulation of egg-laying mediated by the nAChRs. Furthermore, null mutations of gene *C. elegans* only choline acetyltransferase, *cha-1*, are lethal, however, highly deficient alleles (*cha-1* (*p1152*) < 1% of normal levels of ACh) show hyperactive egg-laying, which can be rescued by the expression of CHA-1 in the VCs. This suggests that the VCs play a role in the ACh/GAR-2 mediated inhibition of egg-laying, through its cholinergic connections to HSNs. However, it is also likely stimulatory to egg-laying, as worms that lose both HSNs and VCs are significantly more egg-laying defective than those missing HSN alone. Current models show the HSNs activating the VCs which stimulate the VMs and egg release, but the VCs also form a feedback loop onto the HSNs and inhibiting further egg-laying through GAR-2. The stimulation of an action—egg-laying—as well as a feed back loop within the circuit—VCs inhibition of the HSNs—is an over-represented sub-circuit in the *C. elegans* neurocircuitry and, theoretically, in higher order nervous systems (Reigl et al., 2004).

The action of serotonin on egg-laying is similarly complicated. Serotonin strongly stimulates egg-laying in wild-type animals, even overcoming the inhibition of egg-laying due to the loss of the HSNs—the major serotonergic inputs to the egg-

laying system. However, the further loss of the VCs, in addition to the HSNs, abolishes 5-HT induction of egg-laying (Waggoner et al., 1998). Interestingly, 5-HT induced egg-laying can be rescued in these fully de-innervated animals (HSN⁻/VC⁻) by the addition of exogenous levamisole. Furthermore, worms lacking motor neuron inputs to the egg-laying muscles show high levels of VM activity in response to exogenous 5-HT even though no eggs are laid, similar to the cholinergic response of de-innervated animals (Shyn). The parsimonious interpretation of these data is that 5-HT and ACh form a coincidence detector at the NMJ requiring both signals simultaneously to stimulate egg-laying. However, it should be noted that double mutants of *cha-1*, with almost no ACh, and *cat-4*, defective in the production of both 5-HT and dopamine (*cha-1 (p1152); cat-4 (e1141)*), have no discernable egg-laying defects (Weinshenker et al., 1995). This suggests the prospect of a coincidence detector requiring both molecules simultaneously unlikely, though it does suggest non-cholinergic, non-serotonergic stimulation, such as another signaling molecule or intrinsic activation.

The response to 5-HT is mediated by the five previously described 5-HT receptors. The four GPCRs, *ser-1*, *ser-4*, *ser-5* and *ser-7*, are found in the VMs, but none have been shown to be expressed by the egg-laying neurons (Hapiak et al.; Hobson et al., 2003; Shyn; Xiao et al.). However, any of these receptors may function upstream of the egg-laying system to influence the egg-laying behavior, such as *mod-1*, which is not found in the core egg-laying cells, but has been shown to be required for dopamine induced inhibition of egg-laying (Ranganathan et al., 2000). The loss of

either *ser-1* or *ser-7* does little to native egg-laying rates but abolishes 5-HT's stimulation of egg-laying. However, the further mutation of the *ser-4* and *mod-1* inhibitory receptors causes the worm to regain some response to 5-HT, even in worms defective in both *ser-1* and *ser-7*. Disregarding 5-HT stimulation, mutation of any or all of the 5-HT receptors has little to no effect on the uninduced egg-laying rates or egg-retention (Hapiak et al.; Hobson et al., 2006; Shyn; Xiao et al.). The double mutants *ser-4; mod-1*—both of the inhibitory 5-HT receptors—and the triple mutants *ser-5; ser-1 ser-7*—eliminating the stimulatory receptors—are the most affected in terms of native egg-laying, and even then they are only just statistically significantly abnormal (Hapiak et al.). This highlights the complex and intricate balance of the serotonin signaling involved in egg-laying.

While serotonin stimulates egg-laying and VM activity, it inhibits HSN activity (Shyn et al.). This serotonin induced silencing of the HSNs has only been observed in 5-HT bath assays and might be more important in physiologic conditions without a huge excess of 5-HT present—as the HSNs are the major source of 5-HT in the egg-laying system. HSN silencing requires the $G\alpha_o$ protein *goa-1*, which suggested that *ser-4*, *goa-1*'s 5-HT sensitive GPCR partner, is acting in the HSNs. SER-4 has never been reported as expressed in the HSNs, but it is possible that the expression levels are too low to observe by GFP fluorescence or that it acts on a neuron upstream of the HSNs to elicit this effect. However, several other GPCRs that putatively couple with *goa-1*—*egl-6*, *egl-47* and *gar-2*—are expressed in the HSNs and all have been shown to have an inhibitory role in egg-laying (Bany et al., 2003; Ringstad and

Horvitz, 2008). In addition, *goa-1*'s receptor independent effects have been shown to have lethal consequences in *C. elegans*—though no receptor independent effects have been purported to be involved in egg-laying (Miller and Rand, 2000). This multitude of inhibitory signaling complexes with *goa-1* as common component implies that any one of these could be responsible for 5-HT induced silencing of the HSNs with 5-HT as an upstream initiator.

It is unclear whether all responses to external egg-laying cues are integrated in the HSNs or elsewhere and relayed to the HSNs through neural connections or extrasynaptic signaling. Though it has been demonstrated that the ablation of the neural relays from the HSNs to the head neurons show no effect on egg-laying rates (Zhang et al., 2008). Recently, an extrasynaptic peptidergic pathway was outlined which negatively regulates egg-laying by inhibiting activity in the HSNs. Peptides, *flp-10* & *flp-17*, in response to food are released from neurons in the head with no connections to the HSNs (Ringstad and Horvitz, 2008). They interact with *egl-6*, a GPCR, expressed by the HSNs to inhibit egg-laying in a *goa-1* dependent manner. This is a straightforward example of extrasynaptic signaling controlling egg-laying since the peptides' expression was isolated to neurons without direct connections to the egg-laying system. However, both of these studies were done in experientially poor environments—well feed, optimal conditions—and modulation of these behaviors may be contextual to environmental changes while the basal rates might be stable and unaffected.

In an environment without food, worms will retain eggs but when food is present, they will periodically lay eggs in small bursts (generally 2 – 4 eggs) followed by a return to an inactive state of about 20 minutes (Waggoner et al., 1998). Different signaling molecules in the egg-laying circuit govern the relation between these two states. The loss of ACh signaling in *cha-1* worms dramatically increases the time between egg-laying events in the intracluster time but the inactive period lengths are unaffected, and the loss of 5-HT signaling causes little effect on the intracluster timing but dramatically increases the time spent in between active states. This suggests ACh is responsible for timing during the active, intracluster, state whereas serotonin is a main component in the inactive, intercluster, state. It is also of interest to note that worms missing HSN motor neurons, the most significant source of 5-HT to egg-laying, showed longer intercluster states, as would be predicted, but shorter intracluster periods. In short, egg-laying is a well characterized behavior that may offer insights into NMJ and neuron to neuron communication as well as into multi-molecular signaling networks—ACh, 5-HT, tryamine and peptides—and in small circuit analysis.

Neurophysiology in *C. elegans*

Until relatively recently, physiologic assessment of neurons was unavailable in *C. elegans*. Without cellular physiology and primary biochemistry—dissections of tissues and cell culture, which is still rare in worms—*C. elegans* researchers were almost totally reliant on genetic studies and behavioral analysis to explore the effects

of molecules identified in screens. The addition of cellular physiology techniques allowed researchers to study sensory sensation and intercellular signaling at a new depth and richness. This coupled with the mapped neural connectivity network gave the potential for understanding circuits and even an entire organism. Allowing researchers to couple molecules to cellular responses and further to behavioral outputs, which will eventually combine to build a model of an organism's behaviors.

Electrophysiology

Despite a well characterize and invariant cellular composition, *C. elegans* small size—1mm body length, ~2-3 μ m diameter neurons—and the tough outer cuticle make it particularly challenging to study with electrophysiology. To make matters worse, internal viscera are held under pressure and when the cuticle is compromised they spill forth in an entirely variant manner rendering the cellular positioning diagram useless for the identification of neurons. However, with the advent of GFP to mark neurons of interest, and the efforts of several groups of particularly skilled—and patient—researchers, electrophysiology was adapted to the worm (Richmond and Jorgensen, 1999). The identification of a second nAChR at the BWM is an example of the newly mastered power of electrophysiology. An electrophysiologic study of the BWMs by Dr. Janet Richmond demonstrated a second excitatory receptor that responds to ACh and nicotine, in the absence of the lev sensitive nAChR (Richmond and Jorgensen, 1999). This receptor was later identified as the ACR-16 homomeric nAChR, with little behavioral signature of its own it had been missed for years by

behavioral assays (Touroutine et al., 2005). While the prediction and discovery of new molecules is impressive, it is important to understand that this finding was predicated by the increased sensitivity, and the full benefit of this technique will be the depth of knowledge made available. Electrophysiology continues to unveil the action of molecular forces at the cellular and circuit level with unparalleled richness of molecular information.

Calcium Imaging

However, electrophysiology is not without its drawbacks, importantly, it is a highly invasive procedure which is particularly devastating to the diminutive worm. In addition, only a handful of neurons have been studied with electrophysiology, because it is an extremely demanding technique in terms of time and training, and many neurons are still inaccessible to current dissection techniques. Utilizing *C. elegans* transparency, calcium imaging gives a real-time optical measurement of intercellular calcium concentration allowing neurophysiologic readings live, undissected worms. Activation of a neuron or muscle increases the internal cellular calcium concentration, measuring this influx provides a reasonable proxy for cellular activity. A genetically encoded, calcium sensitive protein, Cameleon, is heterologously expressed in the cell(s) of interest (Kerr et al.; Kerr and Schafer, 2006). Cameleon consists of two fluorophores—yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP)—attached by a calcium sensitive linker. The linker—composed of the calmodulin binding domain and the M13 myosin light chain—changes conformation

in response to the increased concentration of calcium which brings the two fluorophores into close proximity allowing fluorescence resonance energy transfer (FRET) to occur. Light used for excitation only stimulates the CFP, however when in calcium bound conformation FRET transfers this energy from the CFP to the YFP, strengthening the YFP emission and quenching the CFP signal. Comparing simultaneously recorded wavelengths of yellow and cyan yields a ratiometric recording of the calcium levels, and therefore activity, in the cell of interest over time.

$$R_{\Delta T} = [(Y_{\lambda})_{\Delta T} / (C_{\lambda})_{\Delta T}]$$

R = ratiometric change of calcium within the (cell relative), Y_{λ} = YFP emission intensity, C_{λ} = CFP emission intensity

Cameleon is not the only calcium indicator or even the only genetically encoded one, however, it is the only one which provides ratiometric data. Ratiometric recordings filter out movement artifacts and noise that affect both channels equally, giving stable and robust data sets (Kerr et al., 2000).

Cameleon and calcium imaging are not without drawbacks. The response kinetics of Cameleon are the slowest calcium indicating protein or dye, but still on the order of > 100 μ sec to saturation (Miyawaki et al.). Also, expressing heterologous proteins in a live organism is always a concern; even the latest, low-affinity versions of Cameleon chelate enough calcium to affect some behaviors (Dr. Caroline Craig and

Dr. Robyn Branicky, personal communication). Furthermore, the use of calcium as an indicator of activity could also miss important physiologic details within the cell.

Even with these concerns, the areas now opened to *C. elegans* researches by cellular physiology are extraordinary. These techniques open the possibility of detailed study of the cellular circuitry in the worm at a depth well beyond identifying novel molecules and exploring genetic interactions. Egg-laying has been studied for over 30 years, however the binary out-put masks the subtleties involved in the system under the control of so many molecular signaling networks. The monitoring activity of the cells of the egg-laying system with calcium imaging, as done here, will, hopefully, provide a richer understanding of these signaling networks and the interaction among signaling networks. This is highlighted by the finding that in the absence of innervation, serotonin or levamisole will stimulate activity in the VMs, as measured by calcium imaging, however, neither induce egg-laying (Shyn). Here we utilize calcium imaging to examine the effects of serotonin and ACh on egg-laying, in order to elucidate their role in multi-molecular signaling networks. We hope to delineate the function of these receptors in the VMs, the final step in egg-laying, in order to shed light on the process of the molecular and cellular activity involved in egg-laying.

Conclusion

These examples of cellular physiology of BWMs and egg-laying muscles involve muscles directly involved with physical action of the behavior. More complex

efforts are being made to understand the processing of sensory signals in the worm by many *C. elegans* labs including ours. The synaptic connectivity map of the worms and the genetic interaction database built up over the years of research for these behaviors has provided an invaluable resource for cellular physiologists. The ability to trace the effects of molecules through the cellular circuitry and examine the behaviors in terms of the activation of cells, offers fantastic new possibilities for an organism as simple and studied as the worm. These new techniques coupled with the worm's impressive genetic toolbox and connectivity map, give the possibility of understanding the neural network of an entire organism from molecule to behavior.

CHAPTER II

Molecular Mechanisms of Serotonergic and Cholinergic

Control of Egg-laying in *C. elegans*

ABSTRACT

At their most basic level behaviors are controlled by neural molecules that coordinate appropriate actions via complex signaling networks. The *C. elegans* egg-laying behavior is controlled by several molecular cues, including serotonin, acetylcholine and signaling peptides. The cells involved decipher this mélange of signals to coordinate a prescribed and coherent response which we recognize as stereotyped behavior. Though several orders of magnitude more complex, it is theorized that mammalian neurons operate in a similar mixture of molecular signaling to generate behaviors.

Here we use calcium imaging to describe the response of the egg-laying circuit to pharmacological agents in order to understand the molecular signaling networks underpinning its control. We examine the role of serotonin on the vulval muscles directly and in relation to egg-laying behavior. We focus our study on *ser-4*, an inhibitory serotonin receptor. Though, not involved in the initial response to exogenous serotonin, SER-4 maybe at the root several of the contradictory data sets of serotonin signaling with regard to egg-laying. We demonstrate SER-4 to be expressed in the vulval muscles, and from this location, it inhibits vulval muscle activity and modulates long-term response to serotonin.

Furthermore, using a novel dissection technique in combination with calcium imaging, we demonstrate for the first time that acetylcholine can stimulate the vulval muscles directly. This technique will allow future calcium imaging studies to directly

stimulate activity with exogenous agents bypassing the cuticle, which had previously hampered such studies.

INTRODUCTION

Behaviors are a result of a multitude of signaling molecules' response to the environment. The neurotransmitter/hormone serotonin [5-hydroxytryptamine (5-HT)] acts through transmembrane receptors to regulate a multitude of behaviors and processes in both vertebrates and invertebrates, including mood, sleep, muscle activity, as well as cognitive functions (Barnes and Sharp, 1999; de Bono and Maricq, 2005; Roth B.L., 2000). In mammals 13 G-proteins-coupled receptors and a class of ligand-gated ion channels serve to form a complex signaling network of complimentary, antagonistic and apparently redundant components, sometime on a single cell (Hannon and Hoyer, 2008). Further complicating matters, serotonin can function over short distances, pre- and postsynaptically, as well as long distances, in both neurons and non-neuronal cells. Despite its high relevance to many diseases and pathologies, such as depression, anxiety and aggression, the serotonin-signaling network has proved extremely difficult to sort out due to the organizational complexity of the vertebrate systems. Examining multi-cellular signaling networks is a far less daunting challenge in the relatively simple organism *Ceanorhabditis elegans* that also utilizes 5-HT signaling to coordinate behaviors.

The structural simplicity and molecular similarity of worms to vertebrate nervous systems, as well as the wealth of powerful molecular genetic tools available in *C.elegans*, could offer new insights to the building and coordination of a behavioral circuit from molecule to behavior. The *C.elegans* nervous system contains 302 neurons of invariant positions as well as a highly stereotyped and mapped neural connectivity pattern of approximately the same number of chemical synapses as a single human hippocampal pyramidal cell (de Bono and Maricq, 2005). Many behaviors in *C.elegans* are regulated or modulated by 5-HT, including locomotion, feeding, sensory plasticity and egg-laying (Chao et al., 2004; Harris et al., 2009; Ranganathan et al., 2000; Trent et al., 1983). The egg-laying behavior serves as an excellent model because it is extensively characterized, easily quantified, and simple in anatomic structure. Egg-laying is a simple motor program, whose regulation integrates responses to diverse sensory cues such as the presence of food, touch and osmolarity (Avery and Horvitz, 1989; Zhang et al., 2008). The egg-laying circuit is comprised of only 12 core cells, two sets of two motor neurons, and two groups of four vulval muscles (Schafer, 2006). The motor neurons, the hermaphrodite specific neurons (HSNs) and ventral type-C neurons 4 & 5 (VCs), both synapse onto each other and directly onto the vulval muscles (VMs)—see figure 2.1 for more complete description (White J.G., 1986). Egg-laying events show high correlations in firing of both the HSNs and VCs, as well as the coordinated contraction of the VMs resulting in the release of an egg (Zhang et al., 2008).

The simplicity of the circuit and binary output, belies the complexity of the molecular machinery involved in its regulation. Many signaling molecules affect the egg-laying behavior including, but not limited to, 5-HT and acetylcholine (ACh). Both of these signals have been shown to have stimulatory and inhibitory effects on egg-laying depending on the experimental context. (Bany et al., 2003; Brenner, 1974; Schafer and Kenyon, 1995; Trent et al., 1983). Serotonergic control over the egg-laying system is has been characterized by many studies using a variety of tools, pharmacology, as well as ablations of specific cells functioning within the system (Schafer, 2006).

However, the role of serotonin is still unclear, its multifaceted control over the system is difficult to fully define with the binary output of the egg-laying behavior. Calcium imaging is particularly well suited for examining multi-cellular signaling networks with complex and disparate inputs such as 5-HT, because as a non-invasive technique it preserves the physiologic connections and conditions within the organism and provides a rich study of cellular activity (Kerr and Schafer, 2006). We use calcium imaging as well as utilizing *C. elegans* library of mutations and powerful genetic tools such as transgenic expression and cell-specific RNAi knockdowns, to examine the effects of pharmacological agents upon egg-laying and circuit activity. Of particular interest was *ser-4*, which encodes a 5-HT sensitive GPCR, known to be inhibitory to egg-laying (Hapiak et al.; Hobson et al., 2006). We demonstrate that it is expressed in the VM2s—a subset of VMs—and that this expression can directly repress VM activity and inhibit egg-laying. It had been previously reported that SER-4 acts pre-

synaptically, inhibiting the release of ACh, this data would suggest a new mechanism of inhibition by SER-4, acting postsynaptically to repress activity (Nurrish et al., 1999).

It is important to keep in mind that 5-HT is only one of a number of signaling molecules involved with the egg-laying and most other serotonergic mediated behaviors (Komuniecki et al., 2004; Trent et al., 1983). Egg-laying is also under cholinergic control, to understand the interplay between two of the signaling molecules we constructed a microfluidics chamber and utilizing a dissection preparation that allows us to bypass the cuticle to measure the effect of the fast-acting neurotransmitter, ACh, on the VMs. We establish that the VMs are stimulated by ACh and demonstrate that the response is mediated, at least partially, by the *unc-29*, ionotropic nicotinic acetylcholine receptor (nAChR) subunit. Understanding the behavioral implications of such a molecular soup of signals in a small circuit of a discreet output such as the egg-laying system, will provide a powerful platform from which to examine complex signaling regulating behavior and generalize to higher organisms.

RESULTS

Mutation of 5-HT Receptors has no Effect on ACh Stimulation of the VMs

Previous studies have shown that *C. elegans* egg-laying is modulated by at least two stimulatory neurotransmitters/modulators, acetylcholine and serotonin (5-

HT) (Brenner, 1974; Trent et al., 1983). Both of these molecule which are found in the motor neurons innervating the egg-laying circuit, the HSNs and the VCs (Fig. 1) (Duerr et al., 2001; Rand, 1989; White J.G., 1986). Understanding this interaction between the serotonergic and cholinergic signaling systems will be fundamental to understanding the egg-laying behavior and circuit at a molecular level. Nicotine and levamisole, cholinergic agonists, significantly induce egg-laying in wild-type (N2) animals, and levamisole has been demonstrated to increase activity in the VMs (Lewis et al., 1980; Shyn, 2003). These effects are mediated by the nAChRs, whose endogenous agonist is ACh (Kim et al., 2001; Leonard and Bertrand, 2001). However, ACh has never been used as an agonist, due to considerable difficulties in presentation of this agonist to the receptor targets. We developed an assay to assess the effect of acetylcholine on vulval muscle activity directly.

In order to study the effects of ACh, a fast acting neurotransmitter (NT), on the VMs we needed to eliminate the considerable barrier of the cuticle. The cuticle is both a physical barrier as well as a temporal inhibitor disrupting the fast acting neurotransmission we wished to study. To circumvent the cuticle, we used a dissected preparation to have immediate access to the receptors with known concentrations of the pharmacological agents. Using a custom microfluidic device, provided by Dr. Nikos Chronis, which could expose the worm to drugs under tight temporal controls, we examined the VMs response to ACh (see Fig. 2.2). We used a modified *Ascaris* Ringer with high osmolarity (~380 mOsm), which is known to inhibit intrinsic activity in the VMs (Zhang et al., 2008). With this method, we evoked a strong response to 1

mM ACh in N2 worms with a presentation time of 20 msec. [N2 65.7% ($\pm 8.54\%$ SEM)] (Fig. 2.2). This represents the first time *C. elegans* vulval muscle has been directly stimulated by exogenous ACh.

In order to further understand the molecular underpinnings of this response, we attempted to determine the molecules required. As earlier studies had found that mutations in nAChR subunits expressed in the VMs eliminated levamisole induced egg-laying we decided to test one of the nAChR that had displayed this phenotype, *unc-29* (Kim et al., 2001; Lewis et al., 1987a). (UNC-29 is a non-alpha receptor subunit, which causes the total collapse of the heteromeric, levamisole-sensitive nAChR complex in *C. elegans* (Lewis et al., 1987a).) This receptor is also known to be one of two nAChRs involved in excitation of the BWM, whose loss significantly reduces, but does not abolish, the ACh evoked current in the BWM (Richmond and Jorgensen, 1999). Under the same conditions as the wild-type, N2 worms, the evoked activity was abolished in *unc-29 (x29)* mutants [*unc-29* 2.57% ($\pm 1.986\%$ SEM)].

Interested in the potential interaction between ACh and 5-HT signaling we wondered how the lack of 5-HT receptors would affect the response to ACh in the vulval muscles (VMs). Mutants in *ser-1* had shown a resistance to levamisole and nicotine stimulated egg-laying in a previous study leading us to believe that 5-HT signaling might be involved in ACh stimulation (Carnell et al., 2005). This resistance to cholinergic agonist maybe due to communication between the two signaling networks or an overall defect in hyperstimulated egg-laying as they are also resistant to 5-HT induced egg-laying (Xiao et al., 2006). However, ACh evoked activity in

quintuple 5-HTR mutants, which have all five known serotonin receptors mutated, that was indistinguishable from wild type N2 animals [quintuple 5-HTR 62.17% ($\pm 7.96\%$ SEM)] demonstrating that 5-HT signaling is unnecessary for ACh evoked calcium responses in the VMs. This does not rule out the modulation of ACh signaling by 5-HT signaling in the VMs, but does show that the maximal evoked response of ACh is not contingent on 5-HT signaling.

Mutation of all 5-HT Receptors Abolishes 5-HT Induced Activity in the VMs, but Raises Basal Activity

Egg-laying is strongly induced by exogenous 5-HT in N2 animals. Not surprisingly, quintuple 5-HTR mutants are completely resistant to 5-HT induced egg-laying (Hapiak et al.). However, when not stimulated by exogenous serotonin, the egg-laying rate of quintuple 5-HTR mutants is indistinguishable from wild type. These mutants display a mild retention of eggs, but this is a relatively mild defect despite putatively eliminating a highly conserved signaling pathway regulating egg-laying.

We were interested in the cellular activities within the egg-laying circuit mediated by serotonin not apparent in the binary quantification of egg-laying data. Activation of the egg-laying system does not always result in the release of an egg, levamisole can stimulate VM activity in the absence of the HSN motor neurons, but this activity will not result in egg release (Shyn, 2003; Waggoner et al., 1998). We sought to characterize more subtle effects of serotonin upon egg-laying by calcium imaging in order to gain an understanding of its effects at a cellular level. For this

assay, worms were held in a continuous flow of the hypertonic M9 buffer, which is known to inhibit egg-laying, as well as activity in the HSNs, VCs and VMs (Zhang et al., 2008). An initial eight minute recording was made before each trial recording to establish the intrinsic basal activity in the absence of drugs. In N2s, these recordings were marked by a near absence of non-induced or basal activity [0.07 calcium events per minute, (± 0.029 SEM)]. However, even with the high osmolarity and stabilization period there was still significant activity in VMs of the quintuple 5-HTR mutants [0.45 calcium events per minute, (± 0.143 SEM), v. N2 basal rate, $P=0.0044$]. This high baseline of VM activity in the quintuple 5-HTR mutants may account for the phenotypically normal egg-laying rate in native conditions (Hapiak et al.). After the calibration of the basal rate, various concentrations of 5-HT were added to the M9 buffer to stimulate calcium events in the VMs. After a six minute stabilization period, another eight minute recording was made and analyzed. N2 worms showed a significant increase in the frequency at 50 μM 5-HT [N2, Ca^{++} events/min., after baseline correction, 20 μM 5-HT 0.108 (± 0.108 SEM), 50 μM 5-HT 1.46 (± 0.315 SEM), $P=0.016$]. While it has been known for decades that 5-HT stimulates egg-laying and for years that 5-HT stimulates VM activity, these data demonstrate stimulation at concentrations over twenty-five times lower than the previous calcium imaging study (1.3 mM) and orders of magnitude below standard M9 based egg-laying protocols (7.5 – 26 mM) (Dempsey et al., 2005; Hobson et al., 2003; Shyn et al., 2003; Trent et al., 1983; Waggoner et al., 1998). The N2 activity quickly escalated in response to an increasing amount of serotonin. However, the algorithm used to

calculate frequency was saturated after 250 μ M 5-HT, though the pattern of increased response continued (data not shown). The quintuple 5-HTR mutants showed no increase in activity over their high base-line levels, even up to concentrations of 40 mM serotonin—almost 1000X the concentration required to stimulate VM activity in N2s (data not shown).

Basal Activity is not Mediated by an Individual 5-HT Receptor

We were interested in which 5-HT receptors control the basal activity. To investigate this, we studied mutants defective in at a single receptor as well as quadruple receptor mutants, with one functional receptor. (A set of mutants containing only one functional 5-HT receptor, for example a quadruple 5-HT mutant with a functional SER-1 receptor (i.e. *ser-5; ser-4; mod-1; ser-7*), will henceforth be abbreviated *quad (ser-1)*.) We found that mutation of any single receptor did not significantly increase the basal activity observed in the VMs. However, the loss of *ser-7* did significantly reduce the basal activity in comparison to wild-type worms [see Fig. 2.3 B, 0.0089 calcium events per minute, (± 0.0089 SEM), v. N2 basal rate, $P=0.0092$]. This is interesting as *ser-7* functions as an initiator for 5-HT mediated contraction in the pharynx, an extremely low basal rate of spontaneous VM activity in *ser-7* mutant hints that it may act in a similar role in the muscles (Hobson et al., 2006). The basal VM activity in quadruple 5-HTR mutants yielded a similar data set, no combination of mutations caused a basal rate significantly above N2 animals. *Quad (ser-1)* is the only mutant with a significant deviation from the basal activity of these

mutants, and again it displays less activity than the wild-type VM. The increase in basal activity observed in quintuple 5-HTR mutants is not mediated by any one 5-HT receptor and reintroduction of any receptor reduces the basal VM activity to levels observed in wild-type worms.

Mutation in Any 5-HT Receptor Reduces Sensitivity to 5-HT in VMs

Previous studies have demonstrated that the loss of any single 5-HT receptor has little to no significant effect on native egg-laying rates (Carnell et al., 2005; Hapiak et al.; Harris et al., 2009). However, the loss of either *ser-1* or *ser-7*, both expressed in the VMs, abolishes 5-HT induced egg-laying (Hobson et al., 2006; Xiao et al., 2006). The loss of either *ser-4* or *mod-1*, both inhibitory receptors, causes either a moderate increase in stimulation by 5-HT or negligible effects on egg-laying rates depending on the media used in the assay—nematode growth media (NGM) plate-based assays (~140 mOsm) showed a slight increase in mutant egg-laying rates while assays in M9 buffer (310 mOsm) show no difference between N2 and the mutants (Carnell et al., 2005; Dempsey et al., 2005; Hobson et al., 2006). However, mutations of an inhibitory receptor has been shown to at least partially rescue the 5-HT stimulation of egg-laying in both *ser-1, mod-1;ser-1* double mutants, and in *ser-7*, by *ser-4;ser-7* double mutants (Carnell et al., 2005; Hobson et al., 2006). Mutations of *ser-5* are indistinguishable from N2 in egg-laying assays in both native and serotonin induced assays (Hapiak et al.). Seeking to further characterize the effects of these

receptors on egg-laying at a cellular level, we conducted a survey of the mutants in the five known 5-HT receptors on the activation of the VMs by 5-HT.

Here using the assay previously described, we tested the effect of the loss of a single 5-HT receptor upon stimulation of the VMs by exogenous 5-HT. *ser-4* and *mod-1*, inhibitory receptors, showed significant stimulation at 100 μ M 5-HT [Ca^{++} events/min., after baseline correction, *ser-4(ok512)* 0.842 (\pm 0.446 SEM), *mod-1(ok103)* 1.09 (\pm 0.449 SEM)]. *ser-5* and *ser-7* mutant worms showed much less, but still statistically significant stimulation when compared with the quintuple 5-HTR mutants. Surprising considering *ser-7* mutants have been reported to be completely resistant in response to exogenous 5-HT (Hobson et al., 2006). However, VM activity does not necessarily correlate to egg-release (Shyn, 2003). *ser-1* mutants failed to show stimulation at any concentration of 5-HT. Demonstrating the removal of any of the 5-HT receptors diminishes the overall sensitivity of the vulval muscles' response to serotonin.

Each 5-HT Receptor Confers a Unique VM Response to 5-HT

Quadruple 5-HT mutants were tested in a similar manner to assess each receptor's effect on the vulval muscle response in isolation of other receptor inputs. Previous studies observed only minor defects in the native egg-laying rates of the quadruple mutants (Hapiak et al.). All of the quadruple mutants are resistant to 5-HT induced egg-laying, and mutants expressing only an inhibitory receptor, *quad (ser-4)* and *quad (mod-1)*, do not lay eggs in response to 5-HT exposure. In our hands, none

of these quadruple mutants showed activity above the basal rate of the quintuple 5-HTR mutants. In fact, *quad (ser-4)* and *quad (mod-1)* showed significant inhibition from the serotonin when compared with the quintuple 5-HTR mutants, above 100 μM 5-HT, *quad (ser-7)* also showed inhibition at or above 250 μM 5-HT. However, comparing the mutants to elevated basal activity of the quintuple 5-HTR mutants might not be an accurate metric, since adding back any of the serotonin receptors significantly reduces the basal activity observed in the quintuple 5-HTR mutants regardless of the receptor (Fig. 2.2.D).

5-HT Inhibits VM Activity through mod-1 and ser-4

Serotonin can either stimulate or inhibit cellular activity depending on the types of receptors activated. Some of the serotonin receptors have been shown to inhibit egg-laying. We attempted to find if 5-HT can inhibit VM activity through specific 5-HT receptors. Previous studies had found that lower osmolarity buffers induce VM activity in an unknown manner (Zhang et al., 2008). Using a similar setup to the stimulation assay, we induced VM activity with lower osmolarity buffer (40% v/v M9 buffer [~ 125 mOsm]), instead of being inhibited by high osmolarity buffer (100% M9 buffer [310 mOsm]). This induced significant VM activity in N2s and all 5-HT mutants tested, including *tph-1* (a null mutation in tryptophan hydroxylase, required for 5-HT synthesis) (Sze et al., 2000). 5-HT was added to the buffer in order to inhibit the VM activity induced by the low osmolarity. As expected, the added serotonin further stimulated the N2s, as it was above the 50 μM threshold necessary to

stimulate the wild-type worms, and showed no significant effect on the quintuple 5-HTR mutants [Fig. 2.6.A]. The addition of the 5-HT did not stimulate activity in any of the quadruple 5-HT receptor mutants. However, both of the putative inhibitory receptors when expressed in isolation, *quad (mod-1)* and *quad (ser-4)*, showed significant levels of 5-HT induced inhibition of the activity stimulated by the lower osmolarity buffer [Ca^{++} events/min., *quad (mod-1)* native = 4.91 (± 0.988 SEM), 5-HT = 1.14 (± 0.504 SEM), P-value = 0.0041, *quad (ser-4)* native = 6.50 (± 1.08 SEM), 5-HT = 0.438 (± 0.316 SEM), P-value = $8.4e^{-6}$]. These data indicate both *mod-1* and *ser-4* can inhibit the activity of the VMs in response to 5-HT.

SER-4 is Expressed in the VMs

Our lab had previously shown that *ser-4* is expressed in the vulval muscles (Shyn, 2003). Here we include first published pictures of the expression of an integrated *Pser-4::GFP*, *ljIs3*, showing expression specifically in the VM2s. *Pser-4* drives expression in several of other neurons but no expression has been found in either the HSNs or the VCs in this or any study.

VM Expressed SER-4 Mediates 5-HT Inhibition of the VM

We then attempted to delineate the cells in which these molecules are acting in order to inhibit the activity. However, we chose to focus our efforts on *ser-4* rather than *mod-1*, as *mod-1* a) is not expressed in the egg-laying circuit and b) *mod-1* encodes an anion channel, which would inhibit any cell in which it was expressed

giving a high possibility of spurious phenotypes if ectopically expressed (Ranganathan et al., 2000).

As we had data showing *ser-4* is expressed in the VM2s a subset of muscles in the VMs (Shyn, 2003). VM2s are, notably, the only muscles of the VMs to receive significant innervations from the egg-laying motor neurons (White J.G., 1986). Our efforts to identify the cells responsible for the 5-HT induced inhibition mediated by *ser-4*, therefore, focused on the VMs as an integral part of the egg-laying system expressing SER-4. In order to study the VMs specifically, we used the NdE-box promoter, which drives expression in the vulval muscle, as well as the anal depressor muscle and the M4 pharyngeal motor neuron—neither of which are involved with egg-laying (Harfe and Fire, 1998). We used a genetically daisy-chained, nine times repeat of this six base-pair promoter element which induced strong expression in VMs. By expressing SER-4 in quintuple 5-HTR mutants' vulval muscles—both VM1s and VM2s—with an extrachromosomal array ljEx24 [NdE-box(9X)::*ser-4*], we rescued the inhibition found in *quad (ser-4)* animals (see Fig 2.4). Expression of SER-4 in the VMs induced inhibition in mutants that had previously been unresponsive to 5-HT. However, ectopic expression of a receptor followed by application of its agonist may, also, invite spurious results.

To strengthen our hypothesis that SER-4's cellular site of inhibition is the VMs, we looked to eliminate its function specifically in the VMs. By using a cell-specific RNAi technique, we selectively knocked down the expression of SER-4 in the vulval muscles (Esposito et al., 2007). We used the NdE-box promoter again to drive

dsRNA expression of the first 28% of the *ser-4* gene in the *quad (ser-4)* mutants. The inhibition previously shown in *quad (ser-4)* animals was abolished by the knock-down of SER-4 in the VMs. Animals with RNAi depleted SER-4 in the VMs showed no difference in their response to the low osmolarity buffer with 5-HT and the buffer without the drug, similar to the quintuple 5-HTR mutants. The cell-specific RNAi eliminated the inhibition seen in the *quad (ser-4)* animals, as would be predicted if the inhibition was mediated by SER-4 in the VMs. Interestingly, the low osmolarity induced activity in these *quad (ser-4); lJEx276 [NdE-box::ser-4(RNAi)]* animals were similar to that of the *quad (ser-4)* mutants, which is significantly higher than that of the quintuple mutants (see Fig. 2.6.B).

In order to determine if the loss of SER-4 mediated inhibition was mediated in the VMs and not due to ‘spreading’ of the dsRNA to effect other cells in the area we created *quad (ser-4)* animals expressing the same portion of the *ser-4* gene as dsRNA under the *cat-1* promoter, which expresses in the egg-laying motor neurons, the HSNs and the VCs (Duerr et al., 1999). The expression of this construct showed no significant deviation from the *quad (ser-4)* mutants in which it was expressed. This result confirms that the knock-down of SER-4 was specific to the VMs and not a result of the RNAi effect ‘spreading’ as these cells would have been the most likely targets and would have had a similar spreading pattern. With *quad (ser-4)* mutant, extrachromosomal array rescue and the cell-specific RNAi data all in agreement, this strongly point to the VMs as the cellular location for SER-4 mediated inhibition of VM activity.

Adaptation to Serotonin is Mediated by SER-4 in the VM

The *C. elegans*' egg-laying system has also been used to model molecular adaptation to chronic 5-HT exposure (Schafer and Kenyon, 1995). After demonstrating the *ser-4* mediated inhibition of activity in the VMs, we became interested in *ser-4* as a possible molecular mechanism for the adaptation to serotonin. Wild-type worms exposed to a high-level of 5-HT (8.7 mM), after an initial period of hyperactive egg-laying [~ 0 - 4 hour], will adapt by inhibiting egg-laying [+4 hours] retaining eggs (Carnell et al., 2005; Schafer and Kenyon, 1995). The quintuple 5-HTR mutants did not show a significant response to the long term 5-HT exposure, which was expected due to lack of response in all other 5-HT based assays. Mutation in *ser-4*, however, completely abolished the adaptation to serotonin (Fig. 2.8.A). Furthermore, *ser-4* worms continue to lay eggs at a heightened rate in response to the 5-HT. This is not simply at the pre-exposed, naïve rate, as exhibited by the quintuple 5-HTR mutants, since the eggs in the uterus are even younger than the eggs found in *ser-4* non drug-exposed control worms. These data suggest that *ser-4* is responsible for adaptation to chronic 5-HT exposure.

As we had previously demonstrated that the VMs are capable of inhibiting activity in the egg-laying circuit, we decided to focus on this tissue as a likely candidate for mediating adaptation. SER-4 was expressed in the VMs via the NdE-box promoter and tested for 5-HT adaptation. This rescued serotonin adaptation in the quintuple 5-HTR mutants, which lack any response to 5-HT. Demonstrating that VM

expression of SER-4 is enough to mediate serotonin adaptation in absence of other 5-HT inputs.

To further explore this question, we used our previously described cell-specific RNAi constructs to find if knocking down expression of SER-4 in the VMs of wild-type N2 worms was sufficient to abolish adaptation as seen in the *ser-4* mutants. If this were the case, *ser-4* (RNAi) expressed in the VMs should abolish adaptation. We found that, as predicted, wild-type animals expressing the dsRNA *ser-4* construct in the VMs did not adapt to chronic exposure to 5-HT. Moreover, like the *ser-4* mutants, these animals continued to lay eggs at a hyperstimulated rate. Controls expressing this construct in the egg-laying motor neurons of N2 worms display adaptation to 5-HT verifying the authenticity of the VM localized RNAi knock-down of SER-4 result. This demonstrates that *ser-4* expression in the VMs, but not in the motor neurons, is required for the adaptation of egg-laying with 5-HT.

However, *ser-4* mutants expressing SER-4 in the VMs from the same transgene that yielded adaptation in the quintuple 5-HTR mutants display only a small increase in egg-retention. These worms retain significantly more eggs than the *ser-4* worms, however, they do not adaptation to 5-HT and continue to lay eggs at a hyperactive rate (Fig. 2.8A.). These results suggest that the expression of *ser-4* is required but not sufficient for the adaptation to 5-HT and suggests that upstream neurons expressing *ser-4* and other 5-HT receptors are also involved in adaptation of egg-laying to 5-HT.

Single 5-HT Receptors that in Isolation Adapt to 5-HT, Synergistically Combine to not Adapt to 5-HT

Of all of the other 5-HT mutants tested, only *quad (ser-1)* animals were similar to *ser-4* mutants in their failure to adapt to 5-HT. Quadruple 5-HT mutants expressing only *ser-4*, *ser-5*, *ser-7* or *mod-1* all show high levels of adaptation to long-term exposure to 5-HT with egg-retention scores higher than that of N2. While this is unsurprising in quadruple mutants expressing only *ser-4* and *mod-1*, we took this data to mean that the stimulatory 5-HT sensitive GPCRs, *quad (ser-5)* and *quad (ser-7)* molecularly adapt to the high levels of 5-HT. In the case of the *quad (ser-1)* mutants we hypothesized that SER-4 is eliciting inhibitory control over the non-adapting stimulatory SER-1, with the other 5-HT receptors possibly aiding this inhibition.

We became interested in the non-adapting response mediated by *ser-1* and constructed a *ser-4; ser-1* double mutant to further test our model. By our model, the *ser-4; ser-1* double mutants should adapt to chronic 5-HT exposure through the three functional receptors which had showed adaptation, *mod-1*, *ser-5* and/or *ser-7*.

Strangely *ser-4; ser-1* mutants do not adapt to long term 5-HT exposure. This is interesting as all three of the remaining functional receptors, *ser-5*, *ser-7* and *mod-1*, individually show strong inhibition to egg-laying after long-term 5-HT exposure. This would imply that the interaction of these receptors combine to create the opposite phenotype of its constitutive members.

DISCUSSION

Knowledge of a molecule's function and location are fundamental to understanding its role in the regulation of a circuit. To summarize our findings, we a) demonstrate the ACh stimulation of the VMs, b) demonstrate SER-4's ability to inhibit activity in response to 5-HT, c) identify the VMs as a new site of action for SER-4, and d) show that ser-4 is required for egg-laying adaptation to chronic 5-HT exposure. ACh's role in the egg-laying system has been unclear, this is the first time that ACh has been shown to directly stimulate the VMs. These new data also demonstrate the complexity of serotonergic regulation over the egg-laying system and open questions as to the nature and coordination of cellular signaling.

ACh Excites VM Directly Through nAChRs

We show that twenty millisecond bursts of ACh are sufficient to evoke a activity in the VM, and that these responses are mediated by nAChRs. This provides evidence that nAChRs on the VM cell-surface are capable, of stimulating muscle excitation leading to egg release. Nicotine and levamisole, cholinergic agonists, elicit strong stimulation of egg-laying (Trent et al., 1983; Weinshenker et al., 1995). Mutation of nAChR subunits, *lev-1*, *unc-29* or *unc-38*, all known to be expressed in the VMs, cause resistance to levamisole stimulation of egg-laying but cause no appreciable change in native egg-laying rates (Culetto et al., 2004; Fleming et al., 1997; Kim et al., 2001; Lewis et al., 1987a). The levamisole resistance for egg-laying

can be rescued by cell-specific expression of these receptor subunits in the vulval muscles, indicating that nicotinic receptors function directly in neuromuscular synapses (Waggoner et al., 2000a). However, mutants with low levels of acetylcholine (e.g. *cha-1* hypomorphs with <1% normal levels of ACh), lay eggs hyperactively (Bany et al., 2003; Rand, 1989). Furthermore, stimulation of egg-laying by levamisole requires the HSNs (Waggoner et al., 1998). However, a calcium-imaging study demonstrated that levamisole stimulates VM activity even after the loss of both sets of motor neurons, HSNs and VCs (Shyn). In summary, ACh stimulates egg-laying through nAChRs on the VMs, but by itself, this activation is not sufficient for the release of an egg.

A recent study raised further questions about the physiological role of ACh in the egg-laying circuit. They show that raising levels of endogenous ACh in the worm, by mutating the acetylcholine esterase genes, causes retention of eggs (Bany et al., 2003). This effect requires the metabotropic ACh receptor, GAR-2, expressed in the egg-laying motor neurons. This suggests a two level cellular model for ACh control over egg-laying. The motor neurons release ACh onto the VMs to stimulate activity through the nAChRs, meanwhile the ACh released onto these motor neurons, inhibits further ACh release. This model would predict the loss of the ACh mediated inhibition of the motor neurons should allow for hyperactive stimulation in response to exogenous cholinergic agonists, but this is not the case, loss of the HSNs and VCs cause complete resistance to levamisole induced egg-laying (Waggoner et al., 1998). The addition of 5-HT to this assay, however, restores egg-laying. This suggesting the

VMs may act as a coincidence detector for both 5-HT and ACh release. But, this would imply that quintuple 5-HTR mutants should retain eggs, like worms missing the HSNs because this would deprive both animals of the 5-HT required to lay eggs effectively, this is not the case as quintuple 5-HTR mutants do not show an egg-laying defect (Hapiak et al.). Thus, additional factors may be involved in generating egg-laying events in response to cholinergic vulval muscle excitation.

SER-4 Inhibition of VM Activity and Egg-laying

Previous studies have demonstrated *ser-4*'s inhibition of egg-laying and suggested the inhibition of stimulatory neurons upstream of the egg-laying system (Dempsey et al., 2005; Hapiak et al.). Our data demonstrate that SER-4 expressed in the VM can inhibit VM activity, a required step in egg-laying (Schafer, 2006; Zhang et al., 2008). 5-HT mediated inhibition in the VMs creates a much more complex role for 5-HT in the egg-laying system and opens new questions as to the molecular mechanisms underlying serotonergic signaling.

Through its five receptors, 5-HT provides both stimulatory and inhibitor inputs to modulate egg-laying at a variety of cellular levels (Hapiak et al.; Trent et al., 1983). These complimentary and antagonistic signals provide a complex balance in the egg-laying behavior. *C. elegans*' three stimulatory 5-HT receptors, *ser-5*, *ser-1* and *ser-7*, have been demonstrated to be expressed in the VMs (Hapiak et al., 2009; Hobson et al., 2006; Xiao et al.). Mutation in these genes causes little effect in naïve, well-fed animals but both *ser-1* and *ser-7* cause inhibition of egg-laying in response to 5-HT,

while *ser-5* has little effect on 5-HT stimulation of egg-laying. 5-HT induced stimulation of egg-laying can be rescued in these mutants by expressing the wild-type protein in the vulval muscles, identifying the VMs as their site of action. Stimulation of egg-laying by 5-HT can also be rescued by the further loss inhibitory receptor *ser-4* (Hobson et al., 2006). The loss of *ser-4* reduces the overall inhibition 5-HT exhibits on the system, while a *ser-1* mutation reduces the overall stimulation exhibited upon egg-laying, canceling each other's effects and re-balancing the system.

Previous studies had suggested *ser-4* elicited these effects by its activity in upstream neurons whereas the stimulation from *ser-1* and *ser-7* was mediated in the VMs (Hapiak et al.; Hobson et al., 2006; Shyn et al., 2003). However, our results demonstrating *ser-4* expression in the VMs and this as a site of SER-4 mediated inhibition, suggest these antagonistic effects are now acting within a single cell. For a cell to have antagonistic pathways is common, however, to have antagonistic inputs derived from multiple receptors with a common ligand in a non-neuronal cell is a novel finding. It establishes the vulval muscle as a point of integration of 5-HT signaling, blurring the lines between muscle and neuronal function. *Drosophila* larval hearts have been reported to have a bi-phasic response to different concentrations of serotonin, however, this has not been established to be mediated by separate receptors nor is it conclusively antagonistic (Dasari and Cooper, 2006; Zornik et al., 1999). 5-HT mediated inhibition in the VMs—in addition to its already established stimulatory role—creates a much more complex role for 5-HT in the egg-laying system and opens

new questions as to how these signals are integrated by the muscle to produce a coherent out-put.

Vulval Muscle Expressed SER-4 is Required for 5-HT Adaptation

Adaptation is the process by which cells attenuate their response to chronic stimulation. Adaptation at the cellular level is an important mechanism for organisms to respond to their changing environment and is involved in the processing and storage of information (Schafer and Kenyon, 1995). Adaptation of the egg-laying system to 5-HT has been studied as a model for the molecular mechanisms of adaptation. In this work, we show a role novel for *ser-4* in adaptation to 5-HT. This process is, again, centered on the VMs, however, other cells are also implicated in adaptation.

Our findings demonstrate that *ser-4* is required for adaptation to 5-HT. We go on to show that expression of the SER-4 in the VMs is required for adaptation to 5-HT by knocking down expression in the VMs specifically with cell-specific RNAi and demonstrating the loss of adaptation. Furthermore, our results concerning *egl-1* and *ser-4;egl-1* mutants—*egl-1* mutants are genetic ablation of the HSNs motor neurons—demonstrate HSNs motor neurons are not required for *ser-4* mediated adaptation (Desai and Horvitz, 1989). This focuses the adaptation of the VM. Our demonstration of SER-4 mediated 5-HT inhibition provides a potential mechanism for the inhibition of egg-laying as seen in adaptation. This is a novel cellular role for *ser-4* as it had previously been shown to inhibit neurotransmitter release presynaptically (Nurrish et al.).

Previous studies have focused on the motor neurons and described molecules present in the HSNs and VCs required for adaptation (Bany et al., 2003; Schafer and Kenyon, 1995; Schafer et al., 1996). Previous studies that focused on molecules in the motor neurons identified molecules that also conferred hyperactive native egg-laying rates, such as those affecting ACh release and the voltage-gated calcium channel, UNC-2 (Bany et al., 2003; Schafer and Kenyon, 1995; Schafer et al., 1996). In this study, we find expression of SER-4 in the VMs is required but not sufficient to cause adaptation to 5-HT. We show that expression of the SER-4 in the VMs of *ser-4* mutants is not enough to rescue adaptation. Previous studies in our lab have shown that the HSNs are silenced in response to 5-HT, which could be mediated by upstream SER-4 expressing neurons (Shyn et al., 2003). This upstream inhibition could be an additional *ser-4* mediated mechanism of adaptation. These results demonstrating upstream signaling and egg-laying motor neuron involvement in adaptation suggest one of two possibilities a) that many factors are necessary for adaptation, loss of any one of these abolishes adaptation or b) hyperactive egg-laying is not attenuated by adaptation.

Another, extremely interesting result yielded by this study is that three 5-HT receptors individually adapt to serotonin, however, when in combination they no longer adapt. The quadruple 5-HTR mutants, *quad (mod-1)*, *quad (ser-5)* and *quad (ser-7)*, only express a single functional 5-HT receptor; these three quadruple mutants all show strong adaptation to 5-HT. However, *ser-4; ser-1* double mutants, expressing these three putatively adapting receptors in combination, do not show adaptation. The

signaling from separate 5-HT receptors maybe modulating one and other through unknown mechanisms to produce this surprising synergistic effects. The significance of this finding is undetermined as, to our knowledge, this is the first time a phenomenon like this has been reported. This could prove to be an important insight into the modulation among receptors and signaling pathways in *C. elegans* and higher organisms. To this end, *C. elegans* would offer an excellent platform to study this novel mechanism in genetic detail.

MATERIALS & METHODS

Worm Husbandry

The N2 Bristol strain was used as wild-type in this study. *C. elegans* were raised at room temperature (21.5°C) otherwise as describe by Brenner, 1974, on standard nematode growth plates (NGM) agar plates fed with *E. coli* (OP50 strain). All strains, mutants and integrated arrays, were out-crossed a minimum of five times back to the N2 strain before testing.

Young adults were staged by placing several worms (3 – 12, depending on desired density and brood size of strain) on *E. coli* seeded NGM plates overnight then removed the following morning (~14 hours). The worms were grown for 2 days (48 hours) at a room temperature. Larval stage 4 worms (L4s) were selected the day prior to the assay and placed on a new seeded NGM plate and used in assays from 20 – 32 hours after selected as an L4.

Integrated by UV Irradiation

As previously described by Mello & Fire. (Mello and Fire)

Cell Specific RNAi Constructs

Cell specific RNAi experiments were carried out in accordance with protocols outlined in Esposito, *et al.* 2007. The PCR fragments that were used to initiate the RNAi knock-down were constructed as follows. The ser-4 (RNAi) consisted of 488 bp sub-cloned from the lJEx24 plasmid, using primers JC212 (forward) and JC214 (reverse, nested outer). The 9X NdE-box promoter was sub-cloned out the lJEx24 vector—described in Dr. Stanley Shyn’s 2003 thesis—using primers JC204 (forward, nested outer) and JC206 (sense) and JC207 (antisense). Pcat-1 promoter region was cloned out of a genomic DNA preparation with the primers JC208 (forward, nested outer) and JC210 (sense) or JC211 (antisense).

These fragments were fused by PCR in order to create the sense and antisense PCR fragments used to induce the RNAi. The ser-4 (RNAi) was fused to Pcat-1 using JC209 (forward, nested inner) and, either JC212 (antisense) or JC213 (sense, nested inner). This created lJEx277 [Pcat-1::ser-4 (RNAi)], which was used to create lines 3, AQ2510, AQ2511 and AQ2512. The NdE-box promoter was fused to ser-4 (RNAi) PCR fragment using JC205 (forward, nested inner). This created lJEx276 [NdE-box(X9)::ser-4 (RNAi)], which was used to create lines 3, AQ2507, AQ2508 and

AQ2509. Both constructs were injected at a 1:1 ratio of 100 mg/mL using Pmyo-2::mCherry at 5 mg/mL as a marker.

Primer Sequences – uppercase corresponds to promoter sequence lowercase to homologous *ser-4* sequence

JC204 GTAAAACGACGGCCAGT

JC205 AATTTTCAGAGCCTTAAAAATGGCTGA

JC206 GATTGAGAAGCGTCTCGTCGATCacatacctttgggtcctttggc

JC207 CATTTGTTATGTGCAGAATAGGacatacctttgggtcctttggc

JC208 GCTTCAAGGCTCTGCAGGGTACCTATG

JC209 GTACCTATGAATATGCGTAATAGTCG

JC210 GATTGAGAAGCGTCTCGTCGATCgaatgttgattctgaaactgagag

JC211 CATTTGTTATGTGCAGAATAGGgaatgttgattctgaaactgagag

JC212 gatcgacgagacgcttctcaatc

JC213 cctattctgcacataacaaatg

JC214 gaaccaaatacacagtaacatcaacg

Adaptation assay

Adapted from Schafer and Kenyon, 1995 and Schafer *et al.* 1996. Drug plates were made fresh every morning. Serotonin (5-hydroxytryptamine, creatine sulphate complex) (Sigma-Aldrich) was added to melted 1.5% agar (BioGene, UK) and mixed with equal volume of RT water which contained the 5-HT to a final concentration of

8.7 mM. Prior to mixing serotonin was pH balanced to ~ 7.2 pH with sodium hydroxide. *E. coli* was grown over-night, concentrated ($\sim 5X$) by centrifugation and placed on the plates after two hours of hardening and drying. This food was allowed to fully dry before worms were plated. Fifteen to twenty young adult worms were transferred to the test plates.

Young adults in the adaptation assays with *egl-1* worms (N2, *ser-4*, *egl-1*, *ser-4*; *egl-1*, *goa-1*, *goa-1*; *egl-1*) were stage by picking L4s ~ 18 hours before transfer to assay plates. Young adults that did not contain more than ~ 20 eggs were chosen for adaptation assay. This was due to damage sustained by hyperstimulated egg-laying in worms containing significantly above normal eggs in the uterus, resulting in extruded uteruses and other physical defects that artificially impeded egg-laying not related to 5-HT adaptation.

5-HT Stimulation of Activity

Staged young adults were transferred by hair-pick, in order to minimize the amount of bacteria transferred, to a 2% agarose pad (Fisher Brand, Ultra-Pure Agarose) made with M9 buffer. The worms were immobilized upon the pad by gluing their dorsal side to the agarose pad with cyanoacrylate glue (Nexaband SC) leaving the nose and ventral side exposed. A barrier of silicon vacuum grease was built around the agarose pad capable of holding $\sim 1 - 2$ mL of solution. This buffer chamber was then filled with 1 mL M9 buffer to inhibit the activity of the vulval muscles via high osmolarity (310mOsm). The sample was then secured under the microscope (Zeiss

Axioscope, AchroPLAN 63X/0,95w objective) for FRET based calcium imaging of the vulval muscles in accordance with the protocol outlined in Kerr *et al.* (Kerr *et al.*, 2000). A flow of M9 buffer (0.8 – 1.2mL/min.) was started through the grease chamber. The inlet from a polyethylene tube (PE50, Becton Dickton) was fed by gravity and placed 0.2 – 1.0cm above the surface, dripping into the chamber. The drain was a hypodermic needle (18 guage) was attached to a controlled peristaltic pump to regulate the depth of the buffer in accordance to the inflow. After a recording was made—see below for recording schedule—the initial M9 buffer was changed to the test buffer. The test buffer was either unaltered M9 buffer or M9 buffer containing various concentrations of 5-HT. The test buffer flowed from a separate polyethylene tube running in parallel to the first tube also positioned above the buffer chamber (~0.2 – 1.0cm) and allowed to drip into the chamber at a similar rate (0.8 – 1.2mL/min.). This was done so that the subject worm never experienced exogenous 5-HT before the switch occurred, and no contamination was possible. If contact was made between the test buffer outlet and the chamber buffer the preparation was discarded. Furthermore, the aqueous objective was covered with a piece of plastic wrap (Tesco, non-PVC plastic wrap)—a couple of drops of M9 buffer were placed on the objective side of the plastic and secured by a rubber band—in order to eliminate potential contamination of the sample. This plastic wrap and the previously mentioned hypodermic needle were replaced after each assay in which 5-HT was used, the slide with the grease chamber and sample were discarded after every assay.

Transition & Recordings on MetaVue

Three minutes after the flow was initiated, an 8 minute recording of the vulval muscle was made, called the ‘pre’ recording, to establish an intrinsic basal rate of activity before simulation. 1-2 minutes after the pre recording was made the 8 minute ‘switch’ recording was made, at the 2nd minute of the switch recording the initial buffer flow was switched off and the test buffer flow was started. Another 8-minute recording was made immediately after the switch recording called the post recording—~6 minutes after the start of the test buffer. The recordings were analyzed and the frequency of the calcium events were counted. In order to be classified as a separate event the calcium ratio levels must decline to 50% of the baseline of the initiation of the previous event. (i.e. If a calcium influx event starts at baseline (0%) and measures 100% ratio change, the next event will only be counted if the ratio drops back below 50% when it is initiated.) Therefore, if the vulval muscle is stimulated to a high concentration of calcium for an extended for a long period it may not have as high a frequency as a set of smaller events that quickly return to baseline.

Again in accordance with Kerr et al., we analyzed the stack files with jmalyze, and the peaks were identified by an automated computer script run in MatLab as previously described (Kerr et al., 2000; Shyn et al., 2003).

5-HT Inhibition Assay

The set up for the 5-HT inhibition assay was similar to the 5-HT stimulation assay with the exceptions being the pad is a 3% agarose pad made of 50%(v/v) M9

buffer/sterile ddH₂O. Furthermore, the initial buffer that the preparation is bathed in is M9 buffer, same as the stimulation assay, but once the preparation is in position for imaging the buffer solution is changed to a 40%(v/v) M9 buffer/sterile ddH₂O test buffer in order to induced vulval muscle activity by low osmolarity (~128mOsm). After a baseline of activity was established for each genotype assayed another set of tests was run with 100 μM 5-HT. The test buffer was allow to run for 4 minutes then a 4 minute recording (60 frame per second) was taken and analyzed for frequency as previously described.

Calcium Imaging Recording from the Dissected Vulva

This protocol is a modification of the technique described in Kerr *et al.* 2000. Under a buffer of modified Ascaris Ringer with osmolarity raised to 380 mOsm by sucrose glue, using a cyanoacrylate glue (Nexband), young adult worms to a Polydimethylsiloxane (PDMS) (Sylgar 184, Dow Corning) coated cover slip along the ventral side. Two incisions are made posterior and anterior to the vulva, about half the distance from the vulva to the head or tail. At this point, the head should be removed to sever any connections to the nerve ring in the head. The gut and the gonad should be removed. Using the glue, attach a patch anterior and posterior to the vulva to the PDMS coated surface—the glue should be as close as possible without getting glue on the vulva. This will serve to hold the vulva in place for optical recording now that the hydrostatic skeleton of the worm has been compromised.

Once the above dissection is complete, a custom, PDMS microfluidics chamber, provided by Trushal Chokshi of Dr. Nikos Chronis' lab, is placed over the worm while still in buffer. The chamber is brushed with silicon grease to create a seal for the chamber. While still under buffer the chamber is clamped to the PDMS coated cover slip to create a tight buffer-tight seal (PH-1 platform—heating elements removed, Warner Instruments). The secured dissection chamber is transported to an inverted scope (Axiovert, Zeiss) while still under buffer. (Note: unpowdered gloves should be used for all work in buffer to limit the amount of detritus from skin.) Once the tubes to the buffer, drug and vacuums are secured to the microfluidics device, the full device and platform is removed from the buffer in which the worm is dissected and the device is assembled. The chamber is focused, tuned and calibrated for drug delivery by incandescent, bright field light with bromophenol blue dye as a proxy for drug. Flow through the chamber was set between 0.15 and 0.20 mL / second via a peristaltic pump, to minimize mechanically stimulated activity and maximize the speed of clearance of the drug. The acetylcholine was introduced for intervals of 100 μ sec and 250 μ sec during the two minute course of the recording.

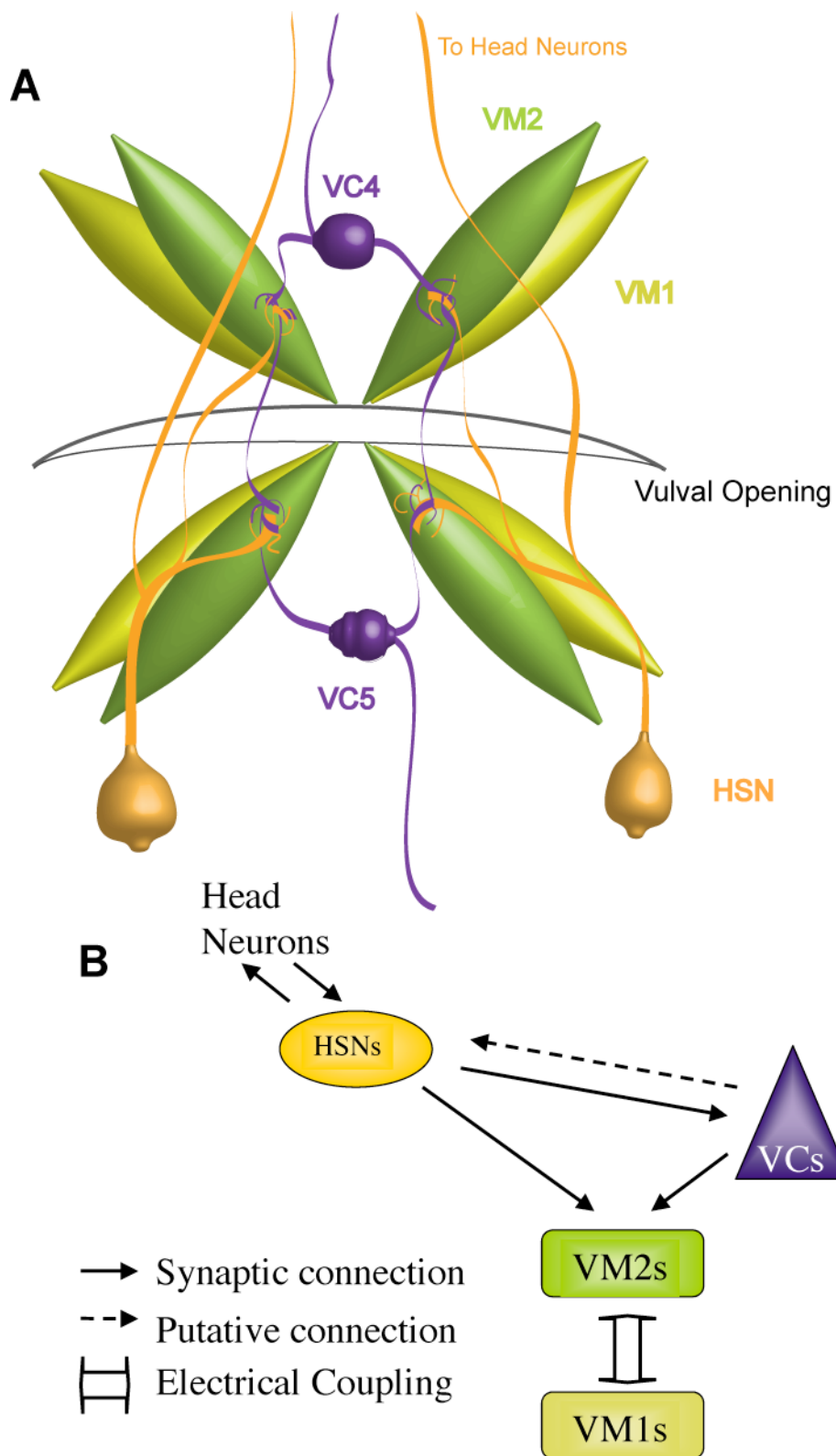
Recordings were possible up to 30 minutes after the dissection of the worm-- up to two hours under slower flow conditions. However, there was a noticeable decline fifteen minutes post dissection, therefore all recording were made twelve minutes after the dissection.

Modified Ascaris Ringer – * denotes modification original recipe

110 mM	Sodium Acetate
23 mM	Sodium Chloride
5.5 mM *	glucose
5 mM	Magnesium Chloride
3 mM *	Calcium Chloride
2.5 mM *	Potassium Chloride
2.5 mM *	Hepes buffer

Analysis of this protocol is described in Kerr, et al. 2000. Only the 100 μ sec stimulation was analyzed, as it was first and consistently larger.

Figure 2.1 Egg-laying System Diagram A) Dorsoventral view of the core egg-laying system (from dorsal to ventral). The principle egg-laying neurons, the hermaphrodite specific neurons (HSNs), are bilateral and posterior to the vulval opening (orange). Only the two most proximal ventral cord type-C neurons, VC4 & 5 (VCs), are depicted (purple). The eight vulval muscles (VMs) are displayed in two groups vulval muscle 2s (VM2s) (more dorsal, green) and vulval muscle 1s (VM1s) (more ventral, yellow). B) The HSNs (orange ellipse) synapse directly onto the VM2s (green box) and onto the other major motorneurons of the system, the VCs (purple triangle). The HSNs also connect to head neurons. The VCs synapse directly onto the VM2s and back onto the HSNs. The VCs also share connections to the other ventral cord type-C neurons (VC1-3&6), which we do not discuss. The VM2s, innervated by both the HSNs and VCs, are electrically couple to the VM1s (yellow rectangle), which have no chemical connections to other egg-laying cells.



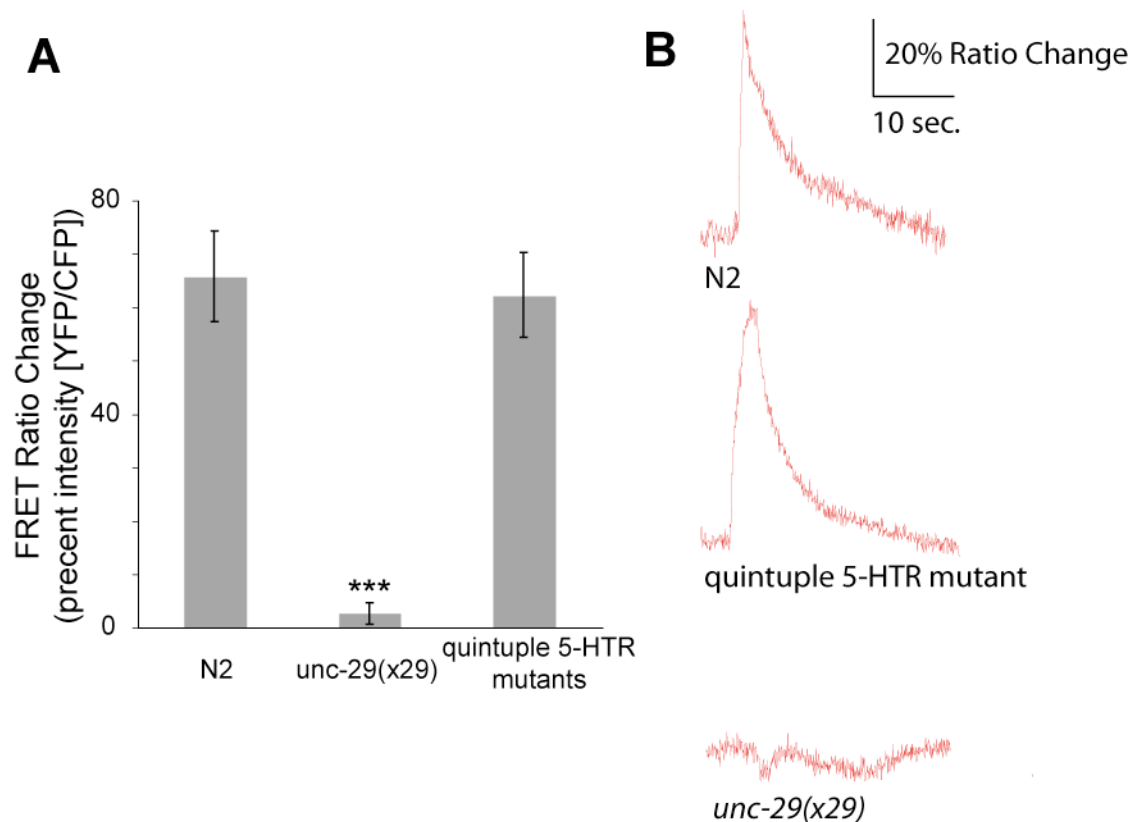


Figure 2.2 VM Stimulation by ACh. Relative strength of calcium influx stimulated by ACh as measured by FRET YFP/CFP ratio change. A) Dissected worms were exposed to 1 mM ACh for 20 mSec which evoke large calcium events in N2 and quintuple 5-HTR mutant worms [N2 65.7% ($\pm 8.54\%$ SEM), quintuple 5-HTR 62.17% ($\pm 7.96\%$ SEM)]. Loss of the levamisole-sensitive nAChR complex, in *unc-29(x29)* mutants, abolishes the response to 1 mM ACh [*unc-29* 2.57% ($\pm 1.986\%$ SEM)]. These mutants displayed significantly less activity than N2, $P=0.00014$, and quintuple 5-HTR mutants, $P=0.00029$. Significance as demonstrated by the student t-test, one-tailed, unpaired: * $P<0.05$, ** $P<0.01$, *** $P<0.001$, [$n > 10$]. B) Representative traces of the ACh induced stimulation.

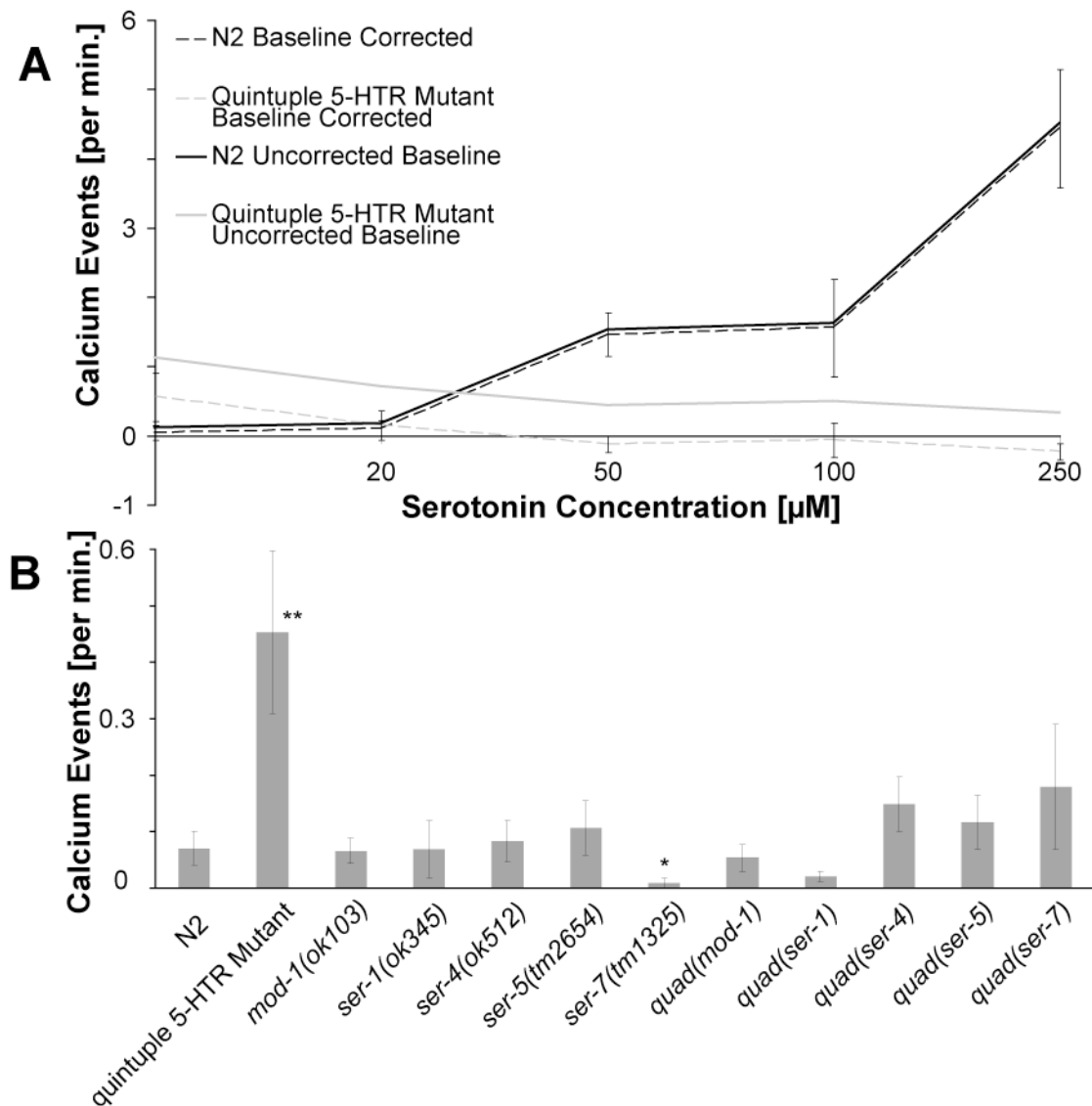


Figure 2.3 Serotonin Strongly Stimulates VM Activity VM activity was suppressed by M9 buffer and stimulated by increasing concentrations of 5-HT the calcium events (\pm SEM). A) 5-HT dosage curves in wild-type (N2) and Quintuple 5-HTR mutants. Solid lines represent the measured activity of the VMs during the experiments; dashed lines represent the baseline corrected for intrinsic activity. [n > 8] B) Basal Activity rates of N2 and 5-HT mutants. The frequency of calcium events in the initial (8 min.) recordings. N2s displayed very low basal activity in high osmolarity M9 buffer where as quintuple 5-HTR mutants significantly elevated basal activity rates. No receptor or combination of 5-HT receptor mutants were as intrinsically active as the quintuple 5-HTR mutants. Significant student t-test, one-tailed, * P<0.05, ** P<0.01, *** P<0.001, [n > 24]. Average served as baseline correction for stimulation assays.

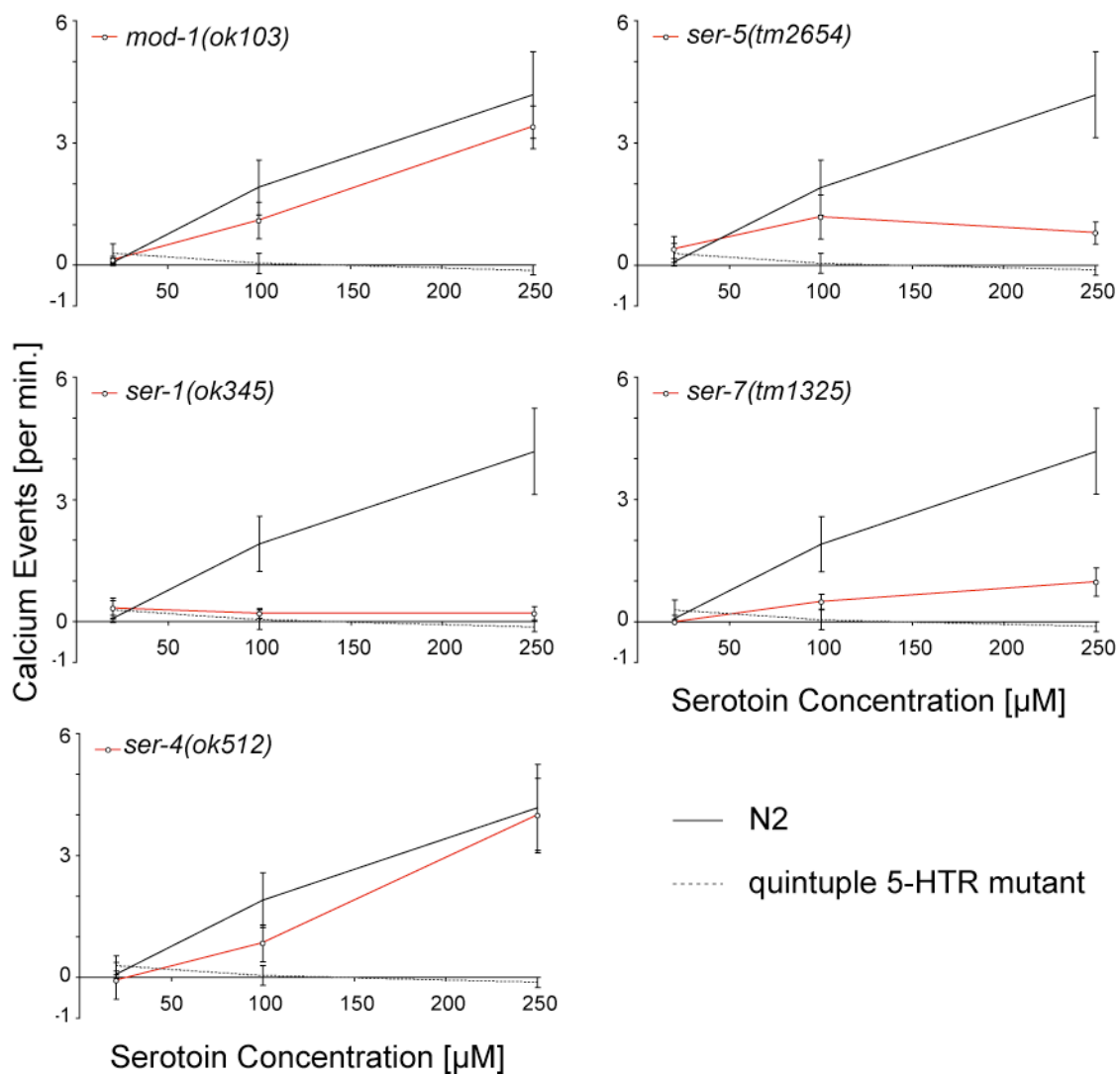


Figure 2.4 5-HT Activity Induced in Single 5-HT Receptor Mutants (baseline corrected) Significant student t-test, one-tailed, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, [$n > 6$].

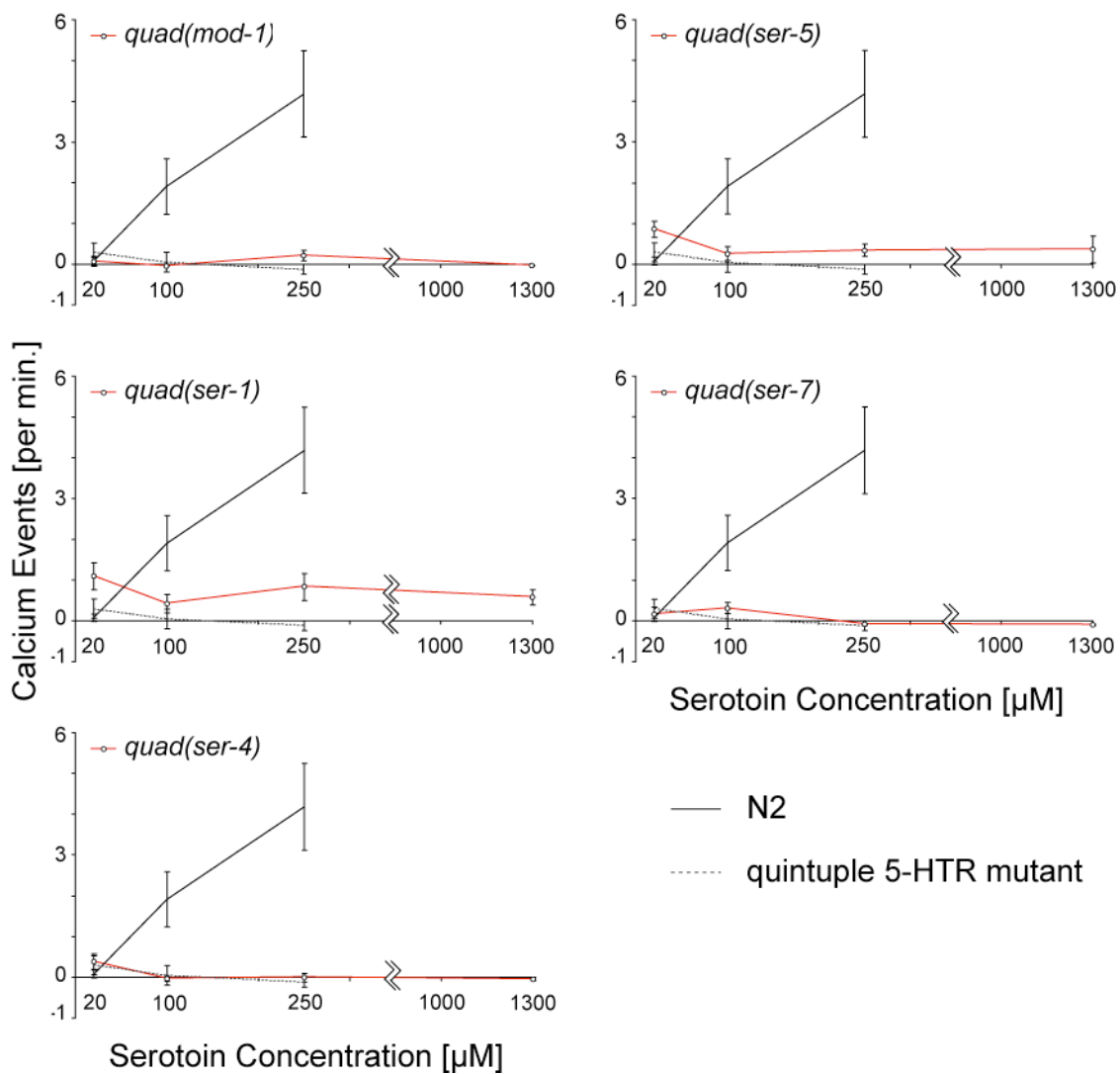
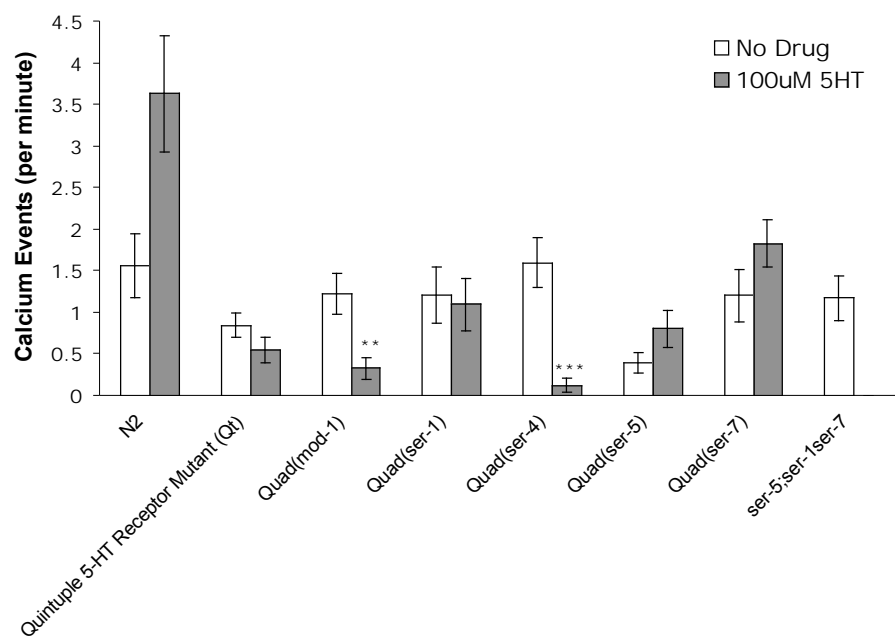


Figure 2.5 Dosage Curve for Quadruple 5-HT Receptor Mutants (baseline corrected) Significance as demonstrated by the student t-test, one-tailed, unpaired: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, [$n > 6$].

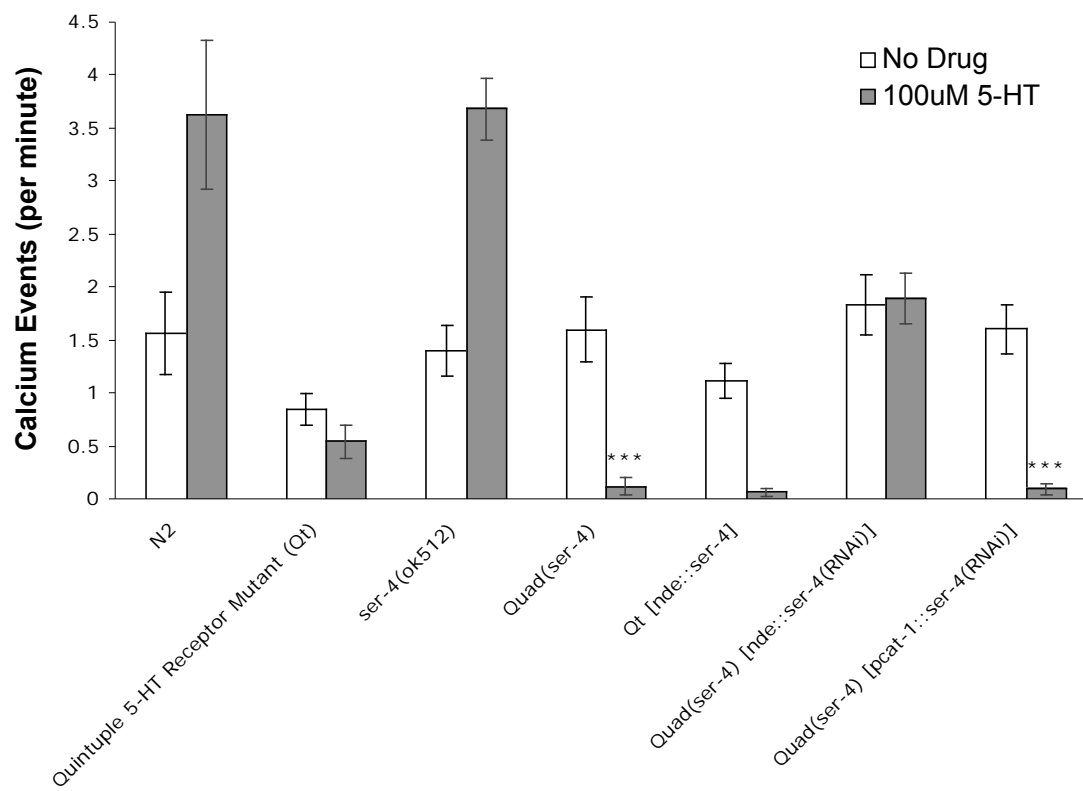
Figure 2.6 SER-4 and MOD-1 Inhibit VM Activity in Response to Serotonin. Worms were exposed to medium osmolarity buffer (40% v/v M9 buffer, ~125mOsm) which induces activity in the VMs. Medium osmolarity buffer containing 100 μ M serotonin was used to inhibit the osmolarity induced VM activity as described in methods. A) *Quad (ser-4)* and *quad (mod-1)* both showed significant reduction in the activity upon introduction of the serotonin. [*quad (mod-1)* P=0.0041, *quad (ser-4)* P=8.4e⁻⁶] [n > 12] B) The expression of SER-4 in the vulval muscles of quintuple 5-HTR mutants rescues the inhibitory response to 5-HT observed in *quad (ser-4)* [P=6.88e⁻⁷]. The expression of a cell-specific *ser-4* (RNAi) construct in VMs of *quad (ser-4)* worms eliminates 5-HT induced inhibition (ljEx276 [NdE-box::*ser-4*]). However, this *ser-4* (RNAi) expressed in the egg-laying motoneurons (HSNs and VC1-6s), showed no effect on *quad (ser-4)* mutants 5-HT mediated inhibition of VM activity (ljEx277 [Pcat-1::*ser-4* (RNAi)]) [P=5.81e⁻⁶].

Significance by student t-test, one-tailed, unpaired: * P<0.05, ** P<0.01, *** P<0.001. [n > 12]

A



B



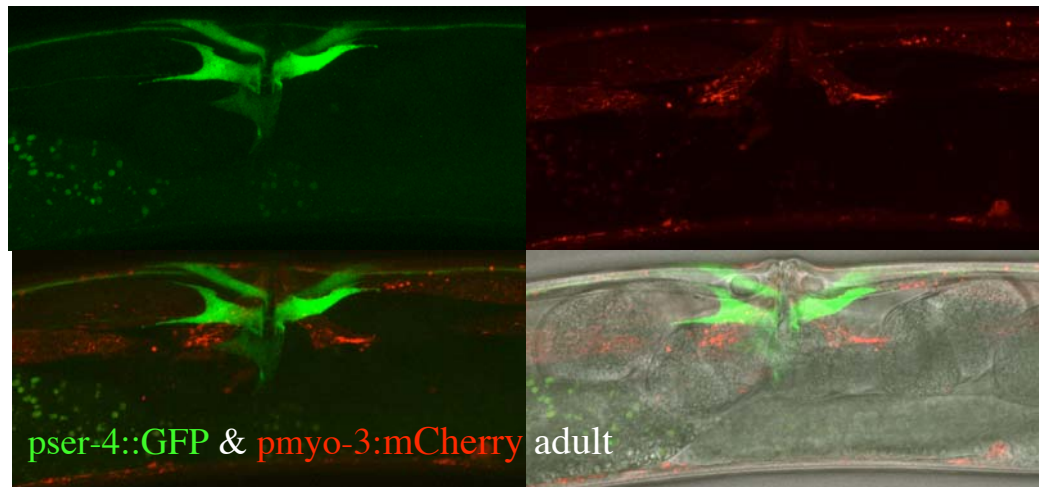


Figure 2.7 Expression of the Integrated Pser-4::GFP, lJIs3. Using Pmyo-3::mCherry as a marker in VMs. Expression of GFP driven by *ser-4* promoter (4.1 kb upstream non-coding sequence) in the egg-laying system is restricted to the VM2s (Green), which are innervated by the serotonergic HSN motorneurons (Shyn, 2003). The Pmyo-3::mCherry is used as a marker for the VMs, and the VM1s can be seen here in red.

Photos courtesy of Vera Hapiak.

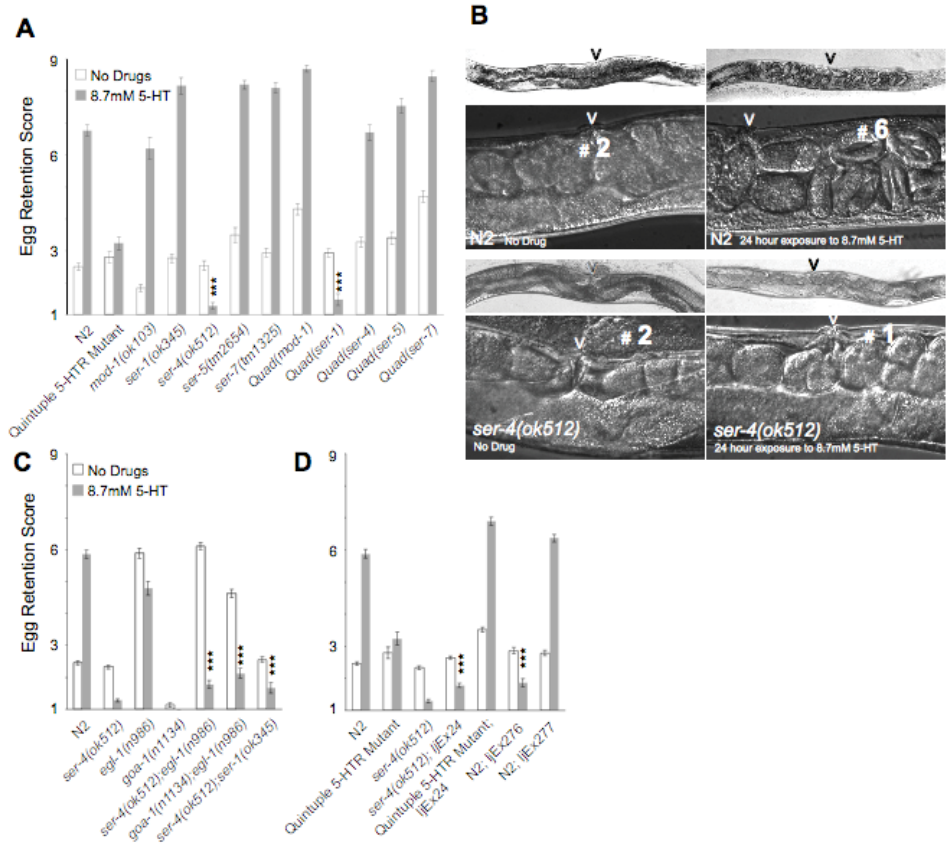
Figure 2.8 SER-4 Expressed in the VMs is Required for Adaptation to Serotonin.

Worms were exposed to serotonin overnight as described in Schafer & Kenyon 1995. N2 animals adapt to long term 5-HT exposure by retaining eggs, scored here according to the most developed egg in the uterus—the higher the score the more developed the embryo/worm. A) N2 worms show adaptation to the 5-HT retaining significantly older embryos in response to 24 hour 5-HT exposure [egg-retention score, no drug 2.50 (± 0.104 SEM), 5-HT 6.54 (± 0.278 SEM), $P = 3.9e^{-26}$]. The quintuple mutants are not affected by the exogenous 5-HT [egg-retention score, no drug 2.80 (± 0.175 SEM), 5-HT 3.13 (± 0.228 SEM)]. *Ser-4* mutant worms are defective in 5-HT adaptation [egg-retention score, no drug 2.53 (± 0.141 SEM), 5-HT 1.23 (± 0.113 SEM), $P = 2.42e^{-9}$] and continue to lay eggs at a heightened rate. Furthermore, *quad (ser-1)* mutants did not adapt to the overnight exposure to 8.7mM 5-HT [egg-retention score, no drug 2.93 (± 0.126 SEM), 5-HT 1.47 (± 0.184 SEM), $P = 2.15e^{-8}$] still laying eggs at an elevated rate as compared to no drug. B) Picture of latest stage egg in N2 and *ser-4 (ok512)* after no drugs or 24 hour exposure to 5-HT. ‘V’ indicates vulval opening, ‘*#’ denotes the latest stage egg in the uterus, with the number indicating the egg-retention score of that egg. (see below.) C) Loss of HSNs, *egl-1 (n986)*, does not affect adaptation [N2 (L4) 5-HT 5.86 ± 0.125 SEM, *egl-1* (L4) 5-HT 4.79 ± 0.215 SEM] (Compared to N2 response for 5-HT adaptation, *egl-1* known to release eggs when exposed to 5-HT, this demonstrates a similar retention of eggs in response to chronic 5-HT exposure (Trent et al., 1983).) *ser-4; egl-1* double-mutants, do not adapt to long-term 5-HT exposure [egg-retention score, no drug 6.10 (± 0.108 SEM), 5-HT 1.77 (± 0.124 SEM), $P = 4.49e^{-5}$]. (Note: all strains in figure used young L4 staging as described in Methods.) D) Expression of SER-4 in VMs of quintuple 5-HTR mutants rescues adaptation, however, *ser-4 (ok512)* still show hyperactive egg-laying after long-term 5-HT exposure. Cell-specific RNAi of SER-4 in the VMs in N2 animals (N2 lJEx276 [nde-box::ser-4 (RNAi)]) abolished the adaptation to 5-HT [egg-retention score, no drug 2.86 (± 0.119 SEM), 5-HT 1.86 (± 0.121 SEM), $P = 5.55e^{-5}$], whereas the same dsRNA construct expressed in the egg-laying motorneurons, HSNs and VC(1-6), had no effect on N2 5-HT adaptation [egg-retention score, no drug 2.88 (± 0.083 SEM), 5-HT 6.37 (± 0.120 SEM), $P = 0.0000512$].

Scoring assignment--higher the score the older the embryo *in utero* (modified from Ringstad & Horvitz, 2008): 1 = 1-8 cell embryo, 2 = 8 - 20 cells, 3 = 21+ cells, 4 = comma, 5 = two-fold, 6 = three-fold, 7 = one worm hatched inside the adult, 8 = 2 - 5 worms hatched, 9 = 5+ worms hatched but adult still alive, 10 = most eggs hatched and consumed by young.

Note: scoring system starts at one, therefore 1 is the baseline for all egg-retention assay graphs.

Significance by student t-test, one-tailed, unpaired: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. [n > 36, over three days for all assays.]



CHAPTER III

High-throughput RNAi Screen for Molecules Involved in Cholinergic Transmission

ABSTRACT

Due to its deleterious effects and widespread use, nicotine is one of the most harmful drugs to global health. However, despite the overall cost to world health, the understanding of molecular mechanisms that mediate nicotine addiction is poor at best. The nicotinic acetylcholine receptors are responsible for the psychoactive properties of nicotine as well as the transduction of cholinergic signaling. We initiated a screen to find novel effectors of the cholinergic signaling system to further our understanding of the molecular elements involved in neurotransmission.

The sinusoidal locomotion of *C. elegans* is based on the coordinated contraction of body wall muscles mediated by cholinergic signaling. Extremely sensitive to perturbations, previous studies of this system have yielded many important molecules in neurotransmission. In this study, we over-stimulate these muscles with a cholinergic agonist, nicotine, causing paralysis. Loss of molecules involved with cholinergic neurotransmission ameliorates the nicotine-induced paralysis. Utilizing an RNAi library to systematically knock down each predicted gene products on chromosome one, we attempt to find new molecules that play a role in cholinergic signaling. Here we identify forty-nine genes which confer nicotine resistance and are specific to cholinergic signalling. It is our hope that these newly identified molecules will provide new pieces to the puzzle of the molecular underpinnings in nicotine addiction.

INTRODUCTION

Tobacco products are the number one cause of preventable death in the US accounting for almost half a million deaths and untold billions in health care costs every year (Danaei et al., 2009). Tobacco use is highly addictive as well as damaging to one's health. The addictive agent, and one of the many pathological chemicals found in tobacco, is nicotine. However, the molecular mechanisms underlying this addiction are still poorly understood. Nicotine elicits its primary psychotropic effect by binding the nicotinic acetylcholine receptor (nAChR). The nAChR is a pentameric, ligand-gated ion channel which when bound to its endogenous ligand, acetylcholine, or an agonist, such as nicotine, allows an influx of cations, Na^+ , Ca^{++} and K^+ , which propagate fast neurotransmission or modulate the function of the cell (Leonard and Bertrand). Past studies have focused primarily on the pharmacology and the cellular expression of nAChRs, which has done little to elucidate the intracellular, molecular mechanisms that mediate addiction (Laviolette and van der Kooy, 2004; Wonnacott et al., 2005). Unfortunately, too few molecules that regulate or modulate nAChR have been identified to substantially map out the intracellular addiction pathway at the molecular level. Our hope was to identify more molecules involved in the expression, regulation and maintenance of functional nAChRs at the cell surface in order to further understand the cellular and molecular processes which underpin addiction.

nAChRs have also been implicated in many other neuronal processes and diseases which have yet to be fully explored. Several diseases, including Parkinson's

syndrome, Alzheimer's disease and schizophrenia include cholinergic signaling as a major component in the current disease models (Kluger, 1996). For example, cell death in Alzheimer's death is caused by β -amyloid induced over excitation of a neuron (Newhouse et al., 2001). However, Alzheimer's patients show a marked cognitive improvement when treated with nAChR agonists such as nicotine. Cholinergic agonists, also, confer neuroprotection from β -amyloid induced excitotoxicity through an unknown mechanism (Kihara et al., 2001). This effect can be suppressed by α -bungarotoxin, a non-competitive nAChR antagonist that prevents activation of the nAChR, suggesting that the neuroprotection is elicited through direct activation of the nAChR (de Fiebre et al., 1995). Another pharmacological study demonstrated the nAChR's involvement in pain sensation (Rashid and Ueda, 2002). Nicotine and epibatadine, another potent nAChR agonist, were identified as effective analgesics, though their mode of action seems to be modulating the (GABA) receptors rather than having a direct effect on nociception. These recent studies have uncovered new roles for nAChRs and opened the door for new therapies targeting the cholinergic signaling system. Discovery and characterization of novel nAChR accessory proteins will provide a more complete understanding of the regulation and functioning of the cholinergic signaling system as it relates to neural pathologies and aid in the development of new treatments.

To this end, *Caenorhabditis elegans* offers an ideal vehicle for the discovery and study of novel molecules in the nervous system. Though simple in design, the signaling and biochemistry of the *C. elegans* nervous system is quite complex and

strikingly similar to higher organisms, even mammals. *C. elegans* utilize almost all of the same neurotransmitters— with the exception of noradrenline—and many modulators/neurohormones (serotonin, dopamine, tyramine, ect.) in common with vertebrates (Horvitz et al., 1982; Komuniecki et al., 2004). Novel molecules involved in neurotransmission found in *C. elegans* often have functionally conserved homologues in mammals, demonstrating the relevance of identifying new neuronal genes in worms. For example, the mammalian homologue of UNC-50, a nAChR trafficking protein identified in *C. elegans*, plays a similar role in mice (Fitzgerald et al., 2000). Furthermore, *ric-3* was identified in a screen for *C. elegans* mutants resistant to acetylcholine esterase inhibitors, and has been demonstrated to play a significant role in the maturation of nAChR in both nematodes and mammals (Halevi et al., 2003). Both of these examples utilized *C. elegans* locomotion as the behavioral metric for reverse genetic screening for neurotransmission abnormalities. Perturbations of such simple, well-characterized systems, such as locomotion, can be identified and traced back to their molecular components, a task made easier in *C. elegans* with its wealth of genetic tools and short generation time.

The addition of RNAi to this already impressive arsenal of genetic techniques has made *C. elegans* even more attractive for novel molecule discovery. RNAi is a double stranded RNA (dsRNA) mediated knockdown of a specific gene's expression. In *C. elegans* this knockdown is systemic, meaning that once the dsRNA is introduced to a few cells the effects will be spread throughout the organism (Boutros and Ahringer, 2008). Feeding *C. elegans* bacteria containing DNA plasmids expressing

dsRNA targeted to a specific gene product can induce this systemic RNAi knockdown (Timmons and Fire, 1998). The RNAi feeding library, created by Dr. Julie Ahringer's lab, is a collection of bacteria expressing dsRNA-targeting constructs targeted at the open reading frames (ORFs), putative genes, of the entire *C. elegans* genome (~86% complete) (Kamath and Ahringer, 2003). [The laboratory of Dr. Marc Vidal has more recently produced a compliment to this library which raises the number of predicted ORFs covered to 98% of the complete genome (Rual et al., 2004).] Specific targeting of the dsRNA constructs offers an important advantage to screens over random mutagenesis: the identity of a positive hit is already known. This saves considerable time over cloning a gene of interest that is identified in a random mutagenesis screen. RNAi offers the combined advantages of a forward genetic screen—ability to select known targets of specific interest—with those of a high throughput reverse genetic screen—absence of bias. This allows researchers to specify and select gene targets or candidate sets of genes, but it is not necessary to do so.

Here we use RNAi mediated knockdown to identify genes involved in the regulation of functional nAChRs. Locomotion is generated by the coordinated contraction of the body wall muscles (BWMs) which is mediated by motor neurons releasing acetylcholine (ACh) on two nAChRs expressed by the BWMs (Chalfie, 1988; Richmond and Jorgensen, 1999). The application of exogenous nicotine causes hyperstimulation of these nAChRs, which causes simultaneous contraction of the BWMs, resulting in a state of rigid paralysis. RNAi knock down of molecules necessary for the functional expression of nAChRs at the cell surface confers

resistance to nicotine induced paralysis, by having less functional nAChRs at the cell surface to be hyperstimulated. Many factors could contribute to impaired nAChR function, such as maturation or modification defects, regulation of receptor expression, general synaptic developmental defects, hyperactive endocytosis, or lack of the receptor sub-units themselves.

Nicotine paralysis was chosen as model for nicotine resistance due to the robust nature of the response, and mutants previously characterized as resistant to cholinergic agonists have been identified as important in cholinergic signaling and highly conserved throughout evolutionarily diverse species, including humans (Eimer et al., 2007; Halevi et al., 2003). The potential for these molecules affecting nAChRs to be relevant to previously mentioned human pathologies was the driving force behind this research. Similar screens have previously been done by random mutagenesis to identify suppressors of levamisole—a nematode specific cholinergic agonist used as an anthelmintic drug—mediated paralysis (Lewis et al., 1980). Several genes including nAChR subunits and important factors, such as the previously mentioned *unc-50*, were identified in these screens (Brenner, 1974). However, the high concentrations of levamisole [1mM] used in these screens meant only worms strongly resistant to levamisole were identified (Lewis et al., 1980). We have attempted to make our screen significantly more sensitive by titrating the amount of drug we use to catch even the smallest perturbation in the regulation of the nAChR. Another important difference is our use of nicotine rather than levamisole. While both cause rigid paralysis, only nicotine affects both of the post-synaptic nAChRs

expressed by the BWMs, and only one is sensitive to levamisole (Richmond and Jorgensen, 1999; Touroutine et al., 2005). This may confer a higher degree of sensitivity to the screen, as a molecule universally affecting nAChRs would act on both and might display a higher level of resistance. A screen very similar to ours was published by Dr. Sieburth and colleagues in *Nature*, again utilizing RNAi as well as the cholinergic agents, levamisole and aldicarb, which inhibits acetylcholine metabolism in the synapse, to test for drug induced paralysis (Sieburth et al., 2005). The remarkable similarity of our protocols served to validate our methods. However, it also ended the novelty of our approach.

RESULTS

RNAi Depletion Confers Resistance to Nicotine-Induced Paralysis

We sought to determine if an RNAi knock-down could confer resistance to nicotine induced paralysis. RNAi is not a complete knock out of the gene target; it is, generally, a 80 – 95% reduction in protein (Boutros and Ahringer, 2008). Unfortunately, this incomplete depletion of a gene product may mask phenotypes seen in the mutants because enough protein is being made to function. This is especially true of proteins with long post-translational life spans, such as the highly regulated and maintained nAChRs (Leonard and Bertrand, 2001). Furthermore, neuronal proteins are also highly resistant to the effects of RNAi for unknown reasons, though our screen is focused on the post-synaptic effects of the nAChR agonist mediated hypercontraction

of the BWM (Simmer et al., 2003; Tavernarakis et al., 2000). Previous studies have shown that worms defective in molecules involved in cholinergic signaling are often strongly resistant to cholinergic agonist induced paralysis, however, this is no guarantee that RNAi depletions will confer similar effects (Lewis et al., 1987b; Lewis et al., 1980). The genes *unc-63* (nAChR β -subunit), *lev-10* (nAChR clustering and maintenance factor) and *unc-50* (nAChR trafficking protein) are important for cholinergic signaling and a mutation in these confers strong resistance to cholinergic agonists (Culetto et al., 2004; Eimer et al., 2007; Gally et al., 2004). We show that RNAi depletion of these genes by feeding dsRNA expressing bacteria will confer significant nicotine resistance (Fig. 3.1). Satisfied with this demonstration of resistance to cholinergic agonists by RNAi knock-down, we sought to further enhance the sensitivity of our screen and increase the speed at which the genome could be probed.

Maximizing the Effect of RNAi

We took several steps in order to maximize the effect of RNAi in our screen. First, we used mutant worms that are hypersensitive to RNAi, *rrf-3(pk1426)* (Simmer et al., 2002). These worms contain a mutation in an RNA-directed RNA polymerase gene, causing hypersensitivity to RNAi. [Note: all worms used in RNAi experiments in this paper are *rrf-3(pk1426)*, including controls which were fed *E. coli* with an empty dsRNA expressing vector.] RNAi enhancer strains, such as this one, increases the effectiveness of RNAi in its ability to knock-down gene products and generate

mutant-like behaviors and have become standard protocol for all RNAi screens since (Boutros and Ahringer, 2008).

Second, RNAi depletion requires the introduction of dsRNA into the worms. There are several protocols designed to accomplish this. We examined each to find the protocol that conferred the greatest resistance to nicotine while still remaining within our logistical capabilities. As such, injecting the dsRNA into each worm was not considered, as this would be far too time consuming for a population-based, genome-wide screening effort that would encompass ~20,000 genes. Two alternatives remained, feeding the worms dsRNA expressing bacteria or soaking the worms in dsRNA containing solution (Kamath et al., 2001; Tabara et al., 1998). Both methods were tested for efficacy in conferring resistance to nicotine with known cholinergic signaling genes (Fig. 3.2). Both protocols showed resistance to nicotine, but neither showed a significant advantage over the other in all but one construct, *unc-29* (a nAChR non-alpha subunit). *unc-29* was strongly resistant in the feeding protocol but conferred no resistance in the soaking protocol. This was most likely due to an error in the sequence of the dsRNA, which either poorly targets or miss targets the dsRNA. This type of miss targeting may occur in either the soaking protocol or bacterial feeding library—as all of the constructs have not been tested individually—and ~7% are targeted to areas of no known genes (Ahringer, 2010). The RNAi soaking protocol has an additional concern in the custom fabrication of the dsRNA itself. Each construct is made in the lab and is therefore subject to human error. Quality testing each dsRNA sequence in parallel with a genome wide-screen would significantly raise

the logistical expense of the screen. Considering both were equally effective and the considerable time and cost advantages of feeding the worms bacteria over the creation of a specific dsRNA construct for each gene, it was decided to use the RNAi feeding library to induce the RNAi knock down.

Furthermore, induction of RNAi via the bacterial feeding library also has multiple potential protocols. The most common and well documented at the time was a protocol developed by Dr. Julie Ahringer's lab in which the worms are exposed over two generations to RNAi in order to maximize the effect (Kamath and Ahringer, 2003). Initiation of the RNAi effect in the maternal worms past down to the progeny strengthens many RNAi induced phenotypes, however, it also may induce sterility and embryonic lethality (Boutros and Ahringer, 2008). This would reduce the number of genes that we could screen, disproportionately eliminating candidates whose mutations cause lethality or locomotion abnormalities so severe as to be missed during previous mutagenesis based screens. This group of lethal and sterile genes is of great interest as it would be eliminated from mutagenesis screens and could yield novel molecules for study. Furthermore, considering that the loss of ACh signaling is fatal, any gene that is universally required for cholinergic signaling is likely to be lethal as well. (It should be noted that the two-generation RNAi protocol was used for initial feasibility studies and nicotine titration studies.)

Initiating the RNAi depletion post-embryonically, introducing the dsRNA after the worms are hatched, had not been established at this point, but offered the possibility to look at the more lethal and sterile genes. We had two different protocols

for this method, a plate based and a liquid-media based protocol. The time, effort and space saving prospects of the liquid-media based protocol were intriguing. However, this protocol was abandoned after the worms grown in this manner failed to move even without the application of nicotine when transferred to the assay plate. The post-embryonic RNAi plate feeding protocol was tested against that of the two-generation Ahringer method and similar resistance to nicotine was found in both methods (Fig. 3.3). The post-embryonic protocol was also much less time and resource consuming than the two-generation protocol (20,000 plates rather than 40,000 plates, and 20,000 less transfers of worms), this coupled with possibility of screening more novel genes led us to use the post-embryonic protocol. (A brief description and depiction of the RNAi induction and primary screening protocol can be found in Figure 3.5.)

Titration of Nicotine to Maximize Valid Hits

In order to maximize the sensitivity of our screen, we titrated the minimum dosage of nicotine needed to elicit the maximal difference in RNAi in treated versus untreated worms. In initial feasibility studies and protocol testing we used 0.5% nicotine (13.3 mM), which was adequate for demonstrating nicotine resistance. However, we could titrate the concentration of nicotine to give us a better chance at discovering novel molecules affecting cholinergic transmission. The ideal concentration would paralyze most, but not all, wild-type worms (~10-20% unanalyzed), while leaving as many of the RNAi treated worms moving as possible. Our screen attempts to identify genes missed by previous mutagenesis screens done

with high concentrations of levamisole, many of these molecules may have been missed because the resistance conferred was too little to be detected. If a certain percentage of wild-type worms are not completely paralyzed, even a low level of resistance should increase this percentage, hopefully to a statistically perceptible level.

In addition, we were interested in a single examination assay time-point that would display maximum nicotine induced paralysis. From previous studies, it was known that maximal paralysis generally occurred 45 to 60 minutes after nicotine exposure, depending on the concentration of drug used, after which the worms start to adapt to the cholinergic agonist (Dr. Alexander Gottschalk, personal communication). While adaptation has significant implications to nicotine addiction, we were interested in resistance in the acute phase of nicotine exposure. These previous studies had been conducted on mutants on agar plates rather than RNAi depleted animals in liquid, so we decided to collect data over 90 minutes to be certain to identify the best time to assay our worms. These tests were run concurrently with our nicotine titration assay.

Using the post-embryonic protocol described above, we tested various concentrations of nicotine on the worms depleted in *unc-63* and *lev-10*. Initially we tested a range of concentrations from 0.1 – 1.0% nicotine but found this range to be far too wide as no worms were paralyzed at the 0.1% concentration and all were paralyzed at the 1.0%. During this test 0.5% nicotine was used as well and showed adequate results. We decided to start with 0.5% nicotine and extend the search from there, we tested 0.3%, 0.4%, 0.5% and 0.6% (Fig. 3.4). After three days of testing, 0.5% nicotine (13.3 mM) proved to be the best choice for our screen, paralyzing all

but 10.6% (± 3.4 SEM) of wild-type worms—within our target range—and displaying large differences between the nicotine resistant controls [unparalyzed at 60 min., *unc-63*(RNAi) 67.7% ± 7.8 SEM, *lev-10*(RNAi) 58.7% ± 9.4 SEM]. During these test, we also found that the best time point to assay nicotine resistance to give maximum separation between empty vector controls and nicotine resistant worms was one hour (Fig. 3.4).

Pilot Screen of Chromosome One

Once the screen was optimized to the highest sensitivity levels and streamlined to maximum high-throughput efficiency, we chose to start our search of the worm genome with chromosome one. We chose chromosome one because it had a high number of genes previously identified as important in cholinergic signaling, such as *unc-38* (nAChR α -subunit), *unc-63* and *lev-10*. Cholinergic signaling genes have a tendency to cluster in regions, as do many genes involved in a single, highly conserved pathway. By coincidence, chromosome one also contained the two genes used as the archetype nicotine resistance response for the initial testing, *unc-63* and *lev-10*, which would serve as verification built into the screen. Chromosome one contains 2,856 predicted open reading frames (ORFs) (confirmed and predicted genes). Of these, 2,416 are represented in the RNAi feeding library that we used (84.6% coverage) (Fraser et al., 2000; Kamath and Ahringer, 2003). We tested 1,837 different RNAi constructs (76.0% of available constructs) (Those not tested were lost

either to contamination of our RNAi bacterial library, or to inviability of some of our bacteria.)

The primary screen was a single pass through the entire chromosome one. In this first round of screening, we used a threshold [32% moving—18 genes not meeting this criterion were chosen from days with low positive controls scores] to classify hits resulting in a large pool of initial candidates (156 or 8.5% of genes tested). This threshold led to a high level of false positives in the empty vector negative control (7.3%). We decided on a low resistance score threshold, accepting a high number of false positives, because the speed and ease of this protocol allowed false positives to quickly be re-tested and eliminated in repeated rounds of screening.

The first round of screening identified genes known to confer resistance to nicotine, *lev-10* [35% (35 moving of 77)] and *unc-63* [51% (23 moving of 45)]. However, another construct putatively targeted to *unc-63* would not have been deemed a hit [29.4%; (10 of 34 moving)], the positive controls for this day were below average, but not significantly. The *unc-63* targeted dsRNA construct identified was the same construct used for initial optimization tests. The first pass screen also failed to identify *unc-29*, a nAChR subunit required for the same levamisole-sensitive receptor complex as *unc-63*, mutations of which are highly resistant to cholinergic agonists (Lewis et al., 1980). Upon further inspection *unc-29* had, unfortunately, not been tested due to contamination or inviability of the bacterial strain when sampled from the glycerol stock for the screen. This should highlight that our screen was essentially blind. The screen was completed using the positional coding that distinguishes each

RNAi bacterial construct in the glycerol library—which has no relation to the gene name—to mark the plates. Only after the second round was complete did we decipher the names of genes that we had identified as resistant to nicotine from their bacterial clone labels.

Re-screening Putative Hits

Considering the high false-positive rate that we had accepted from the initial round of screening, and that it was unlikely that 8.5% of the genes on chromosome one would confer nicotine resistance, we thought it prudent to re-examine these preliminary hits with a second round of testing. This round of screening was identical to the protocol of the primary screen, however, at least three rounds of testing were done with each of the candidate constructs on separate days. With this additional screening, we were able to lower the threshold to characterize the gene as a potential nicotine resistance factor from 29% worms unparalyzed, down from 32% in the primary screen, while decreasing the overall false positive rate. This decrease of threshold increased the false positive rate for the empty vectors [from 7.3% primary screen to 8.2% in the secondary]. However, as the empty vector was examined in triplicate each day, the chance that the vector would be mistaken as an overall hit was ~0.67% and did not occur at any time during these rounds of screening. The 156 candidate constructs that were identified in the first round of screening were augmented by all known and putative neuropeptides, neuropeptide receptors, and GPCRs expressed in the neurons. (By this point, we were aware that this screen was

not to be implemented past the first chromosome and sought to maximize the identification of interesting molecules by cherry-picking potential drug targets—see discussion for further information.) This brought the total number of genes in the secondary screening assay to 195.

Of these 156 identified in the primary screen, forty-three were confirmed as significantly resistant to nicotine after three additional rounds of screening, with an additional six hits in the candidate neuropeptide and neuronally expressed GPCRs. Four of the forty-three nicotine resistant RNAi constructs identified by the screen had no recorded target in the library's database, however, these constructs may still deplete unspecified genes involved in a cholinergic response as only 22 base pairs must align with the target gene in order to complete RNAi mediated knock down. (Note: the dsRNA constructs in the bacteria are often hundreds of bp long (Kamath et al., 2001).) Furthermore, one of these four was identified by blast alignment to be the predicted gene, F59A3.10, though none of the other RNAi constructs had enough similarities across their dsRNA coding region to be identified in this manner. A list of these putative hits and a summary of what is known about each molecule can be found in Appendix I and nicotine resistance scores and functional groupings are in Table 3.1 and Figure 3.6.

Resistance to Non-Cholinergic NT Receptor Agonists

Nicotine resistance could be caused by a number of molecular defects. Secondary screening efforts with other drugs were initiated in an effort to further characterize the effect of the nicotine resistant knock-downs. Muscimol is a GABA

receptor agonist. The GABA receptor is the other major ionotropic channel on BWMs. However, GABA receptors are chloride channels which inhibit the contraction of the muscles (McIntire et al., 1993b). Exposure to muscimol causes complete relaxation of all BWMs and a state of flaccid paralysis in the worm—rather than the contraction of the BWMs and rigid paralysis in response to cholinergic agonists. Muscimol, acting as an agonist against another receptor at the cell surface of the BWM, was used as a control against non-specific drug resistance, such as a less permeable cuticle, and to identify genes universally involved in receptor function (i.e. common to both GABA and ACh receptor function). A highly similar protocol to our plate-based harsh touch nicotine resistance assay was used with 1 mM muscimol substituted for nicotine and a slightly longer time course, ninety minutes. The dsRNA was introduced post-embryonically and young adult worms were transferred to the plates containing 1 mM muscimol. After ninety minutes, the number of worms moving following a slight stimulation with an eyelash was counted. Over 90% of the worms treated with *unc-49* (RNAi) (depletion of *C. elegans* only GABA receptor, UNC-49—positive control) were able to move, whereas only 45% of the empty vector controls were still able to move in response to prodding. Both, *unc-63* (RNAi) and *lev-10* (RNAi) were examined with similar scores to the empty vector control worms, 47% and 30% respectively. Due to the high variability in this assay neither would be considered statistically significant. During these assays, no candidate was significantly different from the empty vector controls, though several, like *lev-10* (RNAi), came close to being hypersensitive to muscimol. These results suggest that we have identified

molecules which are specific to the functional expression of nAChRs, not general resistance factors or universal components of NMJ function.

Nicotine Resistance on Plates

With a little less than fifty potential nicotine resistance factors—forty-five total (thirty-nine from the chromosome one screen and six more from the cherry-picked neuropeptides and receptors) isolated we decided to re-test the worms under the more stringent, but more time consuming plate based assays. The worms were, again, fed dsRNA post-embryonically and raised in accordance to the primary screen protocol. However, for testing, the worms were moved to freshly made (~2-8 hours) NGM plates containing 0.5% nicotine and measurements were taken every 20 minutes up to 80 minutes. Plate-based assays allow the researcher to stimulate the subject worms individually rather than relying on spontaneous movement in animals. This may be a major reason behind the lower variability seen in plate-based assays. This further screening reduced the number of candidate genes to seven, five from the chromosome one screening and two from the neuropeptides and receptors group (Table 3.1). RNAi knock-downs of these genes were consistently more resistant to nicotine over four to six assays. Three of these putative hits were not available as mutants and were categorized as hits based on RNAi phenotypes but no further testing was done, and will be discussed further in the conclusion.

Testing the Mutants for Nicotine and Levamisole Resistance

Deletion mutants of four of the seven putative hits were available from the Caenorhabditis Genetics Center (CGC) or the Japanese National BioResource Project (NBRP) collections and were obtained for nicotine resistance testing. (Two more have become available since we terminated this project.) The mutants available were, *unc-95* and Y6B3A.1 (since re-named: *agef-1*), from the chromosome one screening effort and C48C5.1 and C16D6.2, from the cherry-picked neuropeptide and receptor group. The *agef-1* mutant was homozygous lethal, however, heterozygous mutants were both nicotine and levamisole resistant, but only slightly. It is a putative Arf-GEF, which will be discussed at length in the next chapter of this dissertation. The other three were found to be indistinguishable from wild-type or slightly hypersensitive to cholinergic agonists. *unc-95* is a LIM-domain containing protein which is required for muscle organization. The *unc-95* mutant we received (*unc-95 (ok893)*) was sickly and barely able to move, even without the application of paralyzing cholinergic agonists. Scoring of movement in these worms cannot be considered accurate. The two receptors identified by their RNAi phenotypes also showed hyper-sensitivity to one of the cholinergic agonists but was wild-type to the other. (The C48C5.1 mutant was hyper-sensitive to levamisole and C16D6.2 was hypersensitive to nicotine.) This was likely due to the high homology within the GPCRs and neuropeptide receptors, especially causing erroneous or, more likely, multiple targets for the dsRNA.

DISCUSSION

Our screen identified novel cholinergic resistance factors by RNAi, which we validated through repeated assay trials. The differences in our approach to previous approaches—post-embryonic RNAi based, use of nicotine, minimal drug dosage—yielded a set of putative genes that were not identified in past studies. The differences in phenotype shown by RNAi testing and subsequent mutant trials, highlights the differences between the two screening types and validates our set of putative cholinergic resistance genes as novel.

Genes Identified in the Screen of Chromosome One

The thirty-nine putative nicotine resistance genes that were identified on chromosome one after four rounds of screening fit into a wide range of functional categories. (The GPCRs and neuropeptides were not included on this list as their functions were already known.) Most of these functions are putative and ascribed by homology and protein domain similarity to other known proteins (Fig. 3.6.A & Table 3.1). Four of the largest identified groups [protein synthesis (15%), transcription factors (13%), intracellular trafficking (13%) and degradation (10%)] can be re-classified in a more inclusive category of receptor creation and maintenance (Fig. 3.6.B). These factors would encompass the life-cycle of a receptor, from its initiation to its eventual demise. An abnormality in any of these factors would cause derangement of the expression levels at the cell surface or of the receptors themselves.

The second largest group was the intracellular signaling molecules, composed of G-protein signaling proteins, kinase/phosphatase signaling and molecules with protein/protein interaction domains. While the previous set was involved with the metabolism of the nAChRs, these molecules would likely play a role in the modulation of nAChR function, such as the phosphorylation of receptors to induce inactivation or signals which open cation leak channels lowering activation threshold of the muscle. Structural proteins are also important to neurotransmission and the breakdown of these structures could, also, translate into nicotine resistance. If nAChRs are not clustered at the synapse, as is the case with *lev-10*, a clustering factor, or in a defective synapse, the nAChRs at the cell surface are down-regulated, resulting in less nAChRs at the cell surface to be stimulated, resulting in nicotine resistance (Gally et al., 2004).

Mutant phenotypes

Three of the four genes that conferred significant resistance by RNAi failed to show resistance in the corresponding mutants. While this does not help validate the screen it does highlight some major differences between our RNAi based screen and previous mutagenesis-based searches. With our approach, we sought to differentiate this screen from its predecessors, by use of a novel cholinergic agonist, nicotine, to maximize the identification of molecules that affect both nAChRs present at the BWM. To further differentiate our screen, we used post-embryonic induced RNAi, to minimize the embryonic and developmental effects of the knock-downs and to allow

us to screen lethal and severely locomotion defective mutants that we would not have been able to differentiate from nicotine paralyzed worms.

This was the case with *unc-95*, whose RNAi phenotype we had identified and validated in both the liquid screening efforts and the plate based resistance assays. The *unc-95(su33)* mutants we assayed were almost paralyzed before the application of the nicotine and because there was no base-line re-calibration for severely uncoordinated worms, it was not scored as nicotine resistant. However, UNC-95 represents the type of molecule that our screen was designed to identify: a gene with a defect so severe as to be considered functionally paralyzed even before the application of nicotine in the mutant but able to display a nicotine resistance phenotype in RNAi treated worms. This gene would be missed in a mutagenesis based screen as the innate locomotion defects would mask any potential resistance. Our screening efforts were optimized to find molecules such as UNC-95.

Unfortunately, the literature at the end of the screen identified this molecule as important for muscle structure with severe locomotion defects in the mutants and, therefore, was unlikely to be a molecule involved specifically in cholinergic transmission and was dropped from further study (Zengel and Epstein, 1980). However, since the completion of this screen, new information about *unc-95*'s molecular details have been reported. It was identified in an RNAi based axon guidance screen, along with several other genes that we identified in our screen (Schmitz et al., 2007). It is now known to be involved in axonal fasciculation and guidance as well as the formation of the NMJ, which is critical for neurotransmission

to muscles, cholinergic or otherwise (Broday et al., 2004). The structure of the NMJ is important for maintenance of receptors at the cell surface as several presynaptic components have been shown to be involved in this process (Sanes and Lichtman, 2001). Also, the *UNC-95* protein is heavily expressed in the embryonic and early larval worm, which is a critical time for axonal outgrowth connectivity (Broday et al., 2004). This does suggest that the post-embryonic initiation of RNAi may have saved these worms from some of the more severe effects of the loss of *UNC-95* during embryonic development. In hindsight, an *unc-95* heterozygote or hypomorph (a mutant with less function of a protein, but not a complete, null mutation), which are not nearly paralyzed before the start of the assay, would have been a more appropriate animal to test.

The other two mutants that failed to recapitulate the nicotine resistance phenotype found in RNAi treatments in their respective mutants, C48C5.1 and C16D6.2, are both GPCRs. GPCRs are one of the largest (~5% of all predicted genes), most conserved gene groups in *C. elegans* (Bargmann, 1998). Both have multiple paralogs, resulting from gene duplication events. Within the *C. elegans* genome—C16D6.2 has sixteen paralogs—with greater than 90% sequence similarity (Paulini, 2010). dsRNA only needs 22 base pairs of homology to initiate the RNAi knock-down (Grishok and Mello, 2002). The feeding library initiates RNAi with several hundred homologous base pairs, which gives it a reasonable chance to initiate knock-down in any similar gene much less one with paralogs of over 90% similarity (Fraser et al., 2000). The probability of multiple knock-downs in this case is quite high

and could result in a phenotype ascribed to a single locus even when the phenotype is mediated by the depletion of many similar gene products.

These examples highlight two major differences between RNAi and mutagenesis based screens. First, that RNAi is an incomplete knock-down of the gene product instead of a null mutation, which allows testing of lethal genes. These genes are very difficult to validate with analysis of mutants. Second, RNAi is a targeted tool; the validity of results is based on the precision of the dsRNA. This library was assembled via high-through-put cloning techniques and has a number of known miss targeted constructs. In the release of the RNAi feeding library that we used about 7% (~1300 constructs) are not targeted to a known gene—and even the ones that have listed targets should not be considered absolutely specific (Boutros and Ahringer, 2008; Fraser et al., 2000). With both *unc-95* and the GPCRs, it would have been prudent to test other dsRNA constructs designed specifically against the gene of interest. This would be a way to validate both lethal and non-specific targeting constructs. Unfortunately, we had moved away from the screen, as it was not to be published, and worked on validating only a handful of genes of interest rather than the screen itself.

After the completion of this screen the other nAChR gene mediating cholinergic excitation of the BWM was identified (Francis et al., 2005; Touroutine et al., 2005). These studies demonstrated that in worms the result of the loss of both nAChRs on the BWM is paralysis. This has some unfortunate implications for a screen designed to look at both these nAChRs with paralysis as a metric. Our screen

paralyzes the worms with stimulation to both nAChRs, however, if a gene were to be disrupted that abolished signaling from both of these nAChRs, it would also appear paralyzed. Therefore, this gives the screen an unknown range in which it could find a novel gene: if the resistance factor is not high enough, the worm is paralyzed; if the defect is too great, the worm is also paralyzed.

For a brutal assessment of strategies that could be improved for another RNAi screen and other strategies to optimize an RNAi based screen see appendix 2, 'Getting the most out of your RNAi screen.'

MATERIALS & METHODS

Strains used

The RNAi sensitized strain, *rrf-3 (pk1426)*, was received from the Caenorhabditis Genetics Center at the University of Minnesota.

Pharmacological resistance assays

All plate based resistance assays were done in accordance to the nicotine resistance harsh touch assay as described in Gottschalk, *et al.* 2005. The only changes being the drug and the concentration used, as noted in the text of the dissertation.

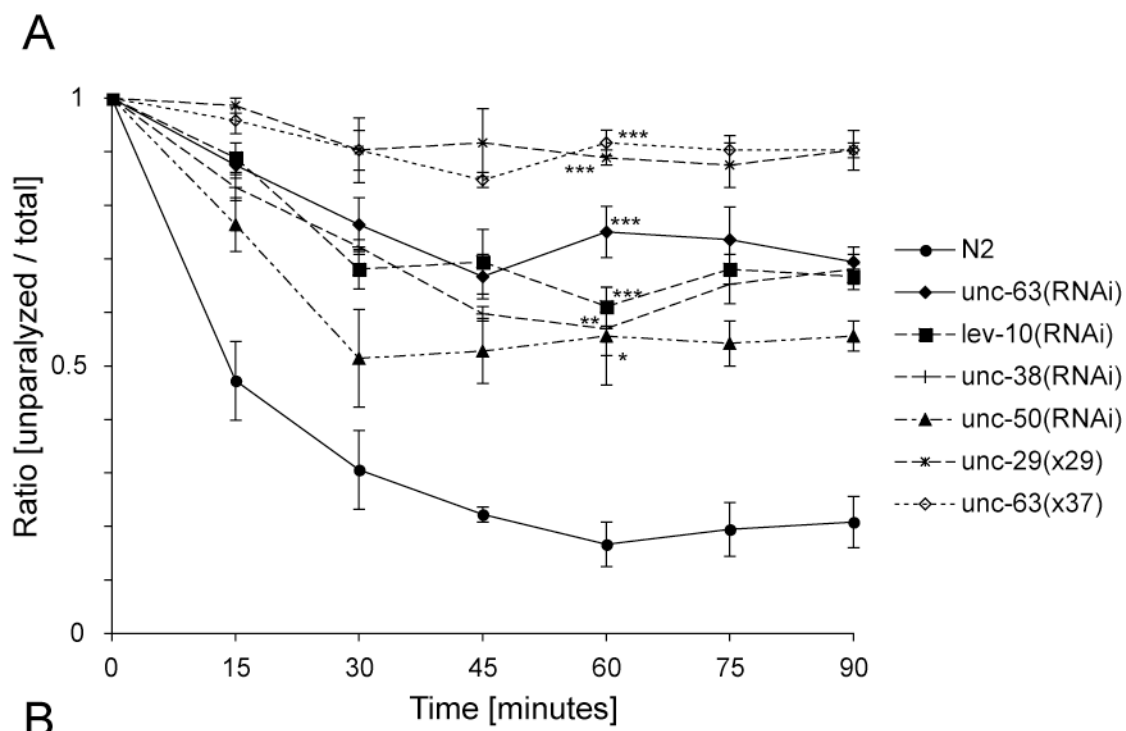
Liquid based protocols were adapted from the thrashing assay described in Gottschalk, *et al.* 2005. In our assays no ngm agarose was present and movement was

scored for movement by eye. The number of worms moving were divided by the total number counted to give a paralysis ratio. The white light source of the dissecting scope was used to stimulate movement. Drugs and concentrations used are noted in the text of this dissertation.

Figure 3.1 RNAi of Cholinergic Signaling Genes Confers Nicotine Resistance

Worms treated with two-generation RNAi against genes involved in cholinergic signaling and known to confer nicotine resistance in mutants were tested against respective mutants and wild-type animals. A) The worms were placed on 0.5% (13.3 mM) nicotine NGM plates as young adults. The number moving (unparalyzed) after mechanical stimulation were counted against the total number of worms observed every 15 minutes for 90 minutes. The in each case the RNAi conferred resistance was significantly above wild-type worms, peaking at 60 minutes. [P-values vs. N2, 60 minutes, *unc-63* (RNAi) = 0.0004, *lev-10* (RNAi) = 0.0007, *unc-38* (RNAi) = 0.0019, *unc-50* (RNAi) = 0.0007] However, the RNAi induced resistance was always significantly less than its corresponding mutant phenotype. B) Pictures of N2 and RNAi treated worms on 0.5% nicotine plates after one hour. RNAi phenotypes demonstrated in *rrf-3* (*pk1426*) animals after two-generation RNAi induction.

n = 6, each n represents one trial of > 24 animals, two trials of each group were run on three separate days. Error bars represent SEM among trials. Significance by Student t-test, one-tailed, unpaired. * = P < 0.05, ** = P < 0.01, *** = P < 0.001. Only the 60-minute time points were considered for statistical analysis and significance.



B

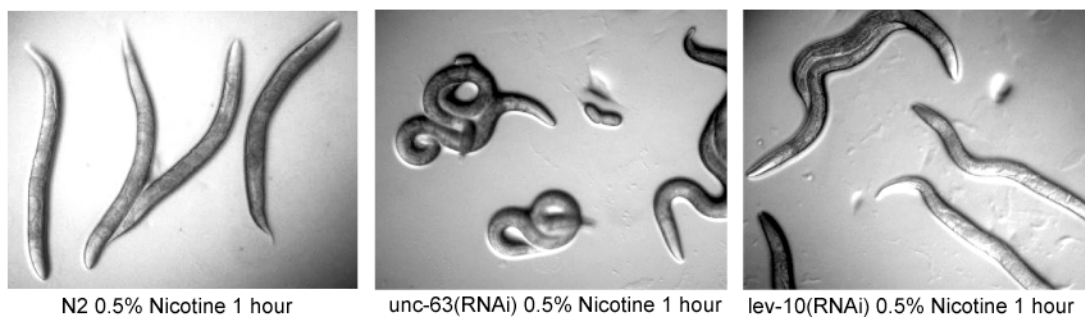
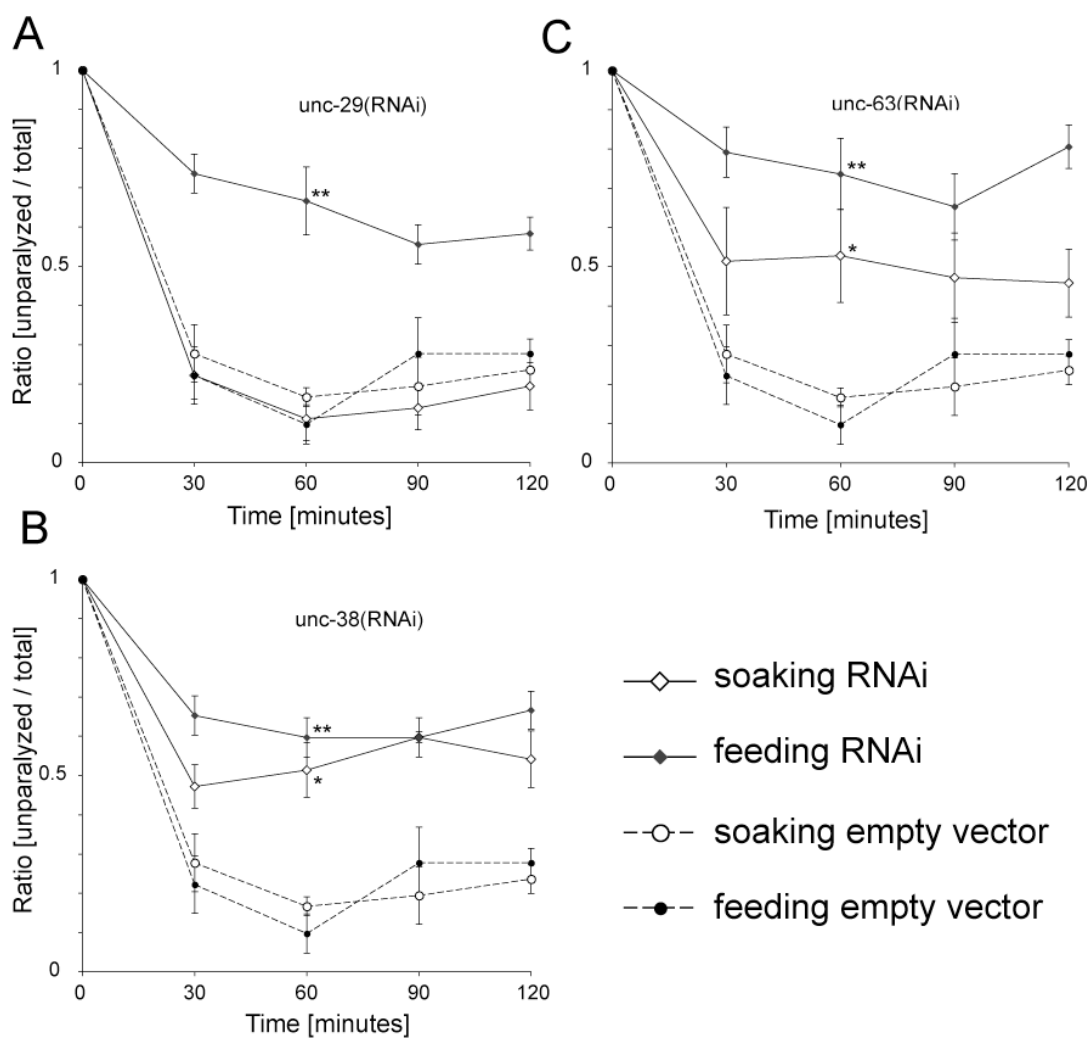


Figure 3.2 Induction of RNAi by Feeding Versus Soaking RNAi effect was induced by either feeding worms dsRNA expressing bacteria for two generations (feeding, solid symbols) according to Fraser et al (2003), or growing the worms in liquid containing dsRNA (soaking, outline symbols) according to Tabara et al (1998). These worms were transferred to 0.5% (13.3 mM) nicotine NGM plates as young adults. The number moving (unparalyzed) after mechanical stimulation were counted against the total number of worms observed every 30 minutes for 120 minutes. A) *unc-29* (RNAi), B) *unc-50* (RNAi) and C) *unc-50* (RNAi); all tested using *rrf-3* (*pk1426*) worms. Control was *rrf-3* (*pk1426*) fed empty vector expressing bacteria (empty vector). Resistance to nicotine conferred by feeding RNAi protocols was significant for every time point. [Feeding, P-values vs. empty vector control, 60 minutes, *unc-63* (RNAi) = 0.0039, *unc-38* (RNAi) = 0.0011, *unc-29* (RNAi) = 0.0045] The soaking RNAi protocol showed significant resistance for both *unc-63*(RNAi) and *unc-50*(RNAi) at 60 minutes, however, *unc-29*(RNAi) failed to induce resistance distinguishable from wild type. [Soaking, P-values vs. empty vector control, 60 minutes, *unc-63*(RNAi) = 0.044, *unc-38*(RNAi) = 0.0136, *unc-29*(RNAi) = 0.216 (not significant)]

n = 5, each n represents one trial of > 20 animals, at least one trial of each animal was run on 4 separate days. Error bars represent SEM among trials. Significance by Student t-test, one-tailed, unpaired. * = P < 0.05, ** = P < 0.01, *** = P < 0.001. Only the 60-minute time points were considered for statistical analysis and significance.



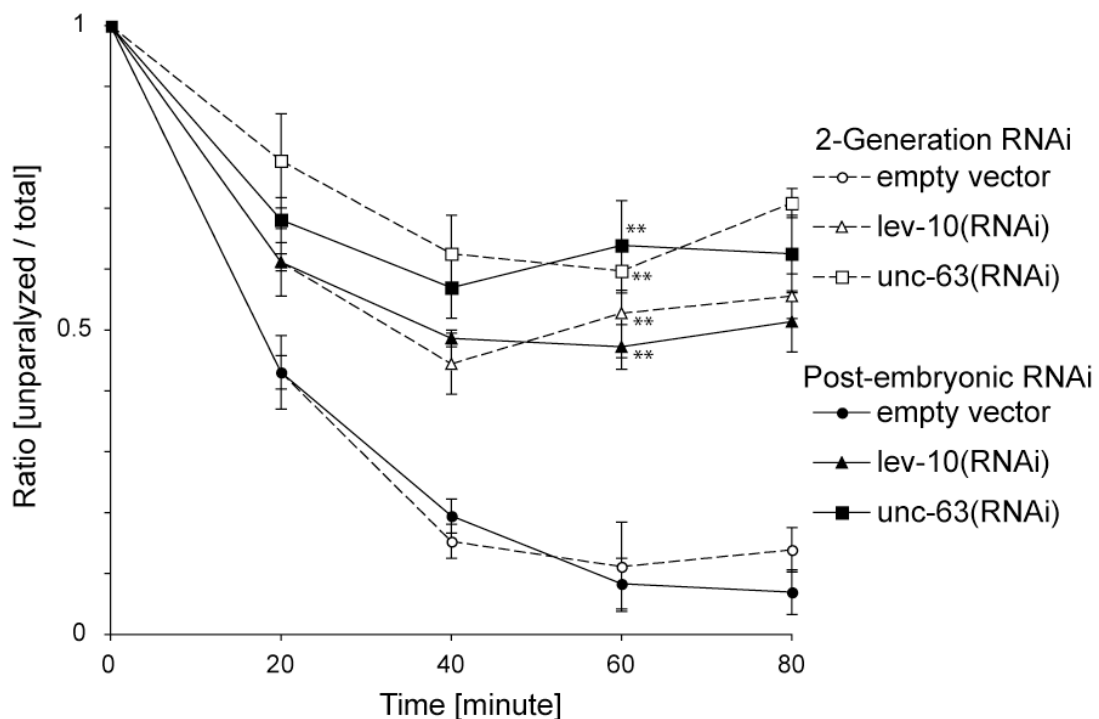


Figure 3.3 RNAi Feeding Protocols: Two-Generation Versus Post-Embryonic Induction *rrf-3* (*pk1426*) worms were feed the same dsRNA expressing bacteria by two different methods. The two-generation RNAi induction (dashed lines, outline symbols) were raised and RNAi induced in accordance with Fraser et al (2003). The post-embryonic RNAi induction (solid lines, solid symbols) protocol is described here in Materials and Methods. (Also, see Fig 3.4. for diagram and more explanation.) After RNAi treatment the worms were placed on 0.5% (13.3mM) nicotine NGM plates as young adults. The number moving (unparalyzed) after mechanical stimulation were counted against the total number of worms observed every 20 minutes for 80 minutes. No significant differences was noted between nicotine resistance induced by either protocols, both protocols induced significant resistance in comparison to empty vector controls of same protocol. [P-value vs. empty vector, 60 minute; two generation, lev-10 (RNAi) = 0.0080, unc-63 (RNAi) = 0.0051; post-embryonic, lev-10 (RNAi) = 0.0012, unc-63 (RNAi) = 0.0030]

n = 3, each n represents one trial of > 24 animals, one trial of each animal was run on three separate days. Error bars represent SEM among trials. Significance by Student t-test, one-tailed, unpaired. * = P < 0.05, ** = P < 0.01, *** = P < 0.001. Only the 60-minute time points were considered for statistical analysis and significance.

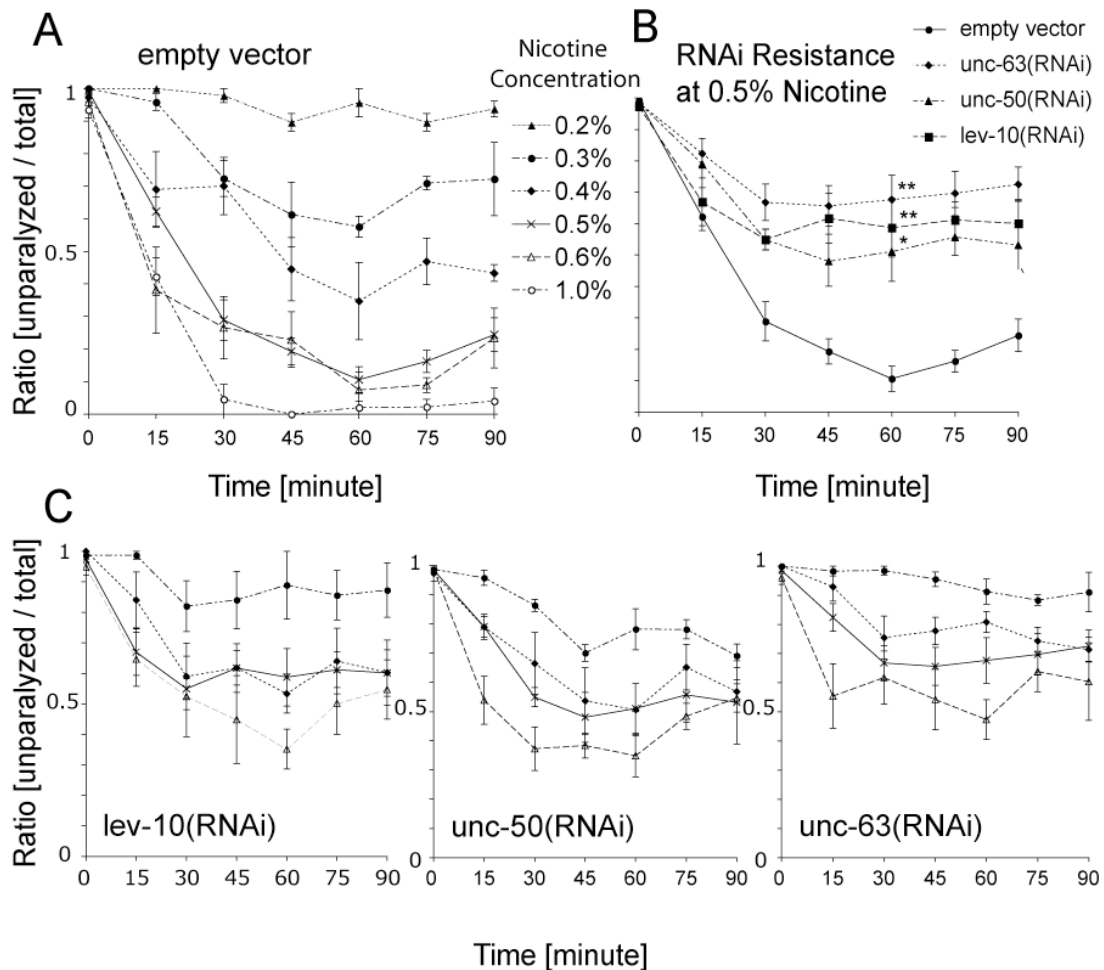


Figure 3.4 Determination of Nicotine Dosage Post-embryonic RNAi was induced in *rrf-3* (*pk1426*) worms, which were transferred to M9 buffer containing various concentrations of nicotine [0.2% - 1.0 %]. The number moving (unparalyzed) spontaneously were counted against the total number of worms observed every 15 minutes for 90 minutes. A) *rrf-3* (*pk1426*) fed empty vector bacteria tested on various concentrations of nicotine [0.2% - 1.0%], B) *lev10* (RNAi), *unc-50* (RNAi) and *unc-63* (RNAi) assayed at 0.5% nicotine against empty vector control worms. All three tested constructs showed significant resistance to nicotine at all time points after 30 minutes. [0.5% nicotine, P-value vs. empty vector, 60 min.; *lev10* (RNAi) = 0.0076, *unc-50* (RNAi) = 0.0213, and *unc-63* (RNAi) = 0.0058]. C) *lev-10*(RNAi), *unc-50* (RNAi) and *unc-63* (RNAi) resistance at various concentrations of nicotine [0.3% - 0.6%].

For all trials except trials done at 0.5% nicotine (panel 'B') $n = 3$, each n represents one trial of > 24 animals, one trial of each animal was run on 3 separate days. Error bars represent SEM among trials. For 0.5% nicotine trials: $n = 6$, one trial a day on 6 separate days. Significance by Student t-test, one-tailed, unpaired. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

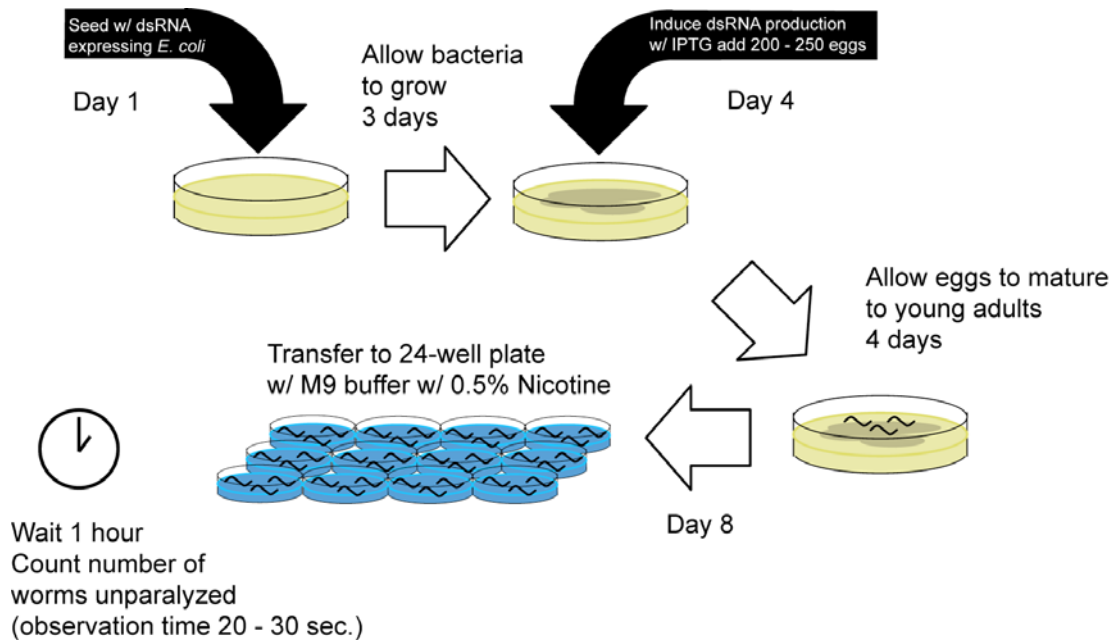
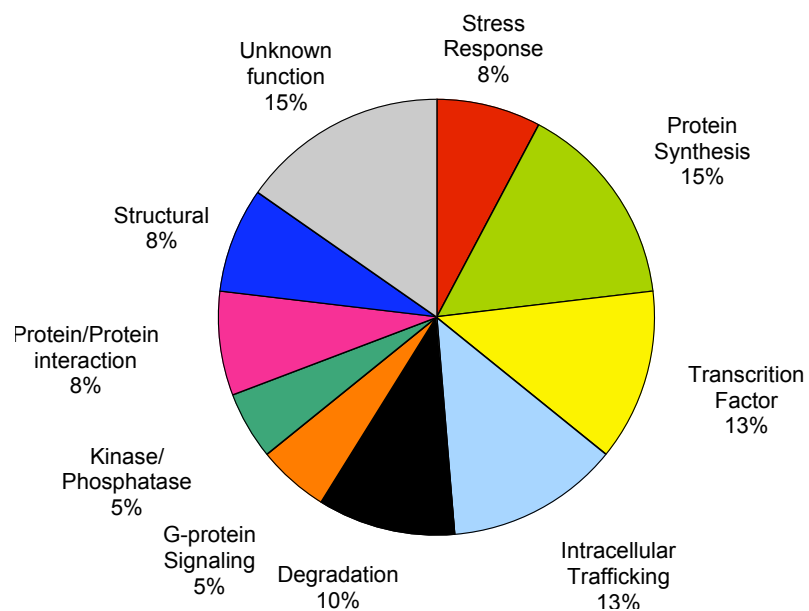


Figure 3.5 Post-Embryonic Induction of RNAi and Nicotine Resistance Assay *E. coli* expressing dsRNA constructs are grown in a 96-well plate overnight (37°C) and seeded to NGM plates, one plate per dsRNA strain. The dsRNA expressing bacteria NGM plate is allowed to grow for an additional three days at RT. Concurrently, large quantities of *rrf-3* (*pk1426*) are grown on separate plates. The eggs from these are harvested by standard bleaching protocols and washed and suspended in M9 buffer. The M9 buffer egg slurry is diluted to ~250 – 300 eggs / 100 μ L of solution including the addition of IPTG, to induce the production of the dsRNA, to a final concentration of 2.5 mM. 80 μ L of slurry was added to each plate and spread over the entire bacterial patch, to ensure even induction of the dsRNA expression by the IPTG. The eggs were allowed to mature to young adults, 4 days, RT. 25 – 30 worms were moved to a 24-well plate containing ~2 mL of M9 buffer and 0.5% (13.3 mM) nicotine and allowed to incubate for one hour. Worms were assayed under dissecting microscope for spontaneous movement. The number of moving worms (unparalyzed) were counted against the total number of worms observed.

Figure 3.6 Putative Functions of the Nicotine Resistance Factors Functions and putative functions of genes identified as resistant to nicotine by estimated domain identity and homology (Paulini). A) Specific putative functions of nicotine resistance factors. B) Broader grouping of categories. (See table 3.1 and appendix 1 for more information on specific nicotine resistance factors.)

A



B

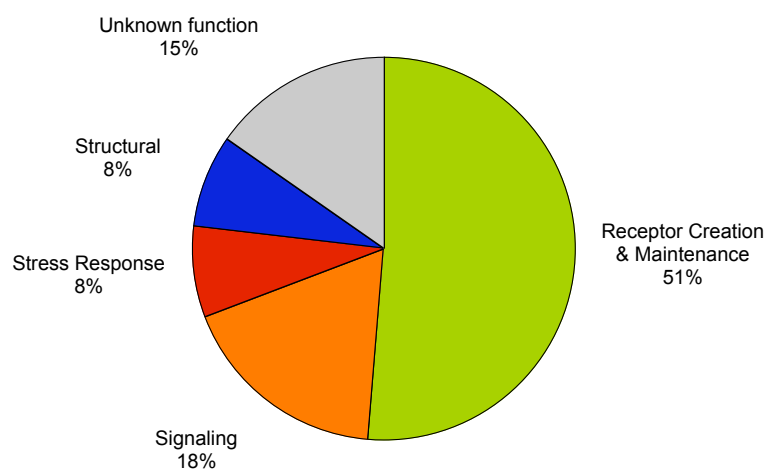


Table 3.1 Putative Functions of Genes Mediating Nicotine Resistance Forty-nine dsRNA clones and their corresponding genes found to be resistant to nicotine exposure after four rounds of nicotine paralysis assays. Selection criteria > 32% unparalyzed during the primary assay, > 29% unparalyzed during three subsequent rounds of nicotine resistance testing. Scores listed are an average of all four tests—one during primary screening, at least 3 more in subsequent rounds—also listed is the first standard deviation of this data. ‘Group’ describes the function or putative function of the molecule based on literature or domain homology; colors and titles refer to figure 3.6. Seven genes highlighted in yellow demonstrated significant resistance in at least six plate based assays. (See appendix 1 for details on genes described.)

Gene name	Score (Average)	Standard Deviation	Group
srh-49, C10G11.4	0.32	0.14	G-protein signaling
D2030.2	0.38	0.16	Degradation (Ubiquitin & Protease)
C30F12.2	0.34	0.20	Metabolism/mitochondria
gsp-3, W09C3.6	0.36	0.13	Phosphatase & Kinase
col-61, C01H6.1	0.46	0.13	Structural
hrdl-1, F26E4.11	0.31	0.09	Degradation (Ubiquitin & Protease)
fbxb-56, Y63D3A.10	0.35	0.05	Protein-protein interactions
C10H11.8	0.33	0.18	G-protein signaling
agef-1, Y6B3A.1	0.45	0.16	G-protein signaling
W03G9.5	0.35	0.03	Phosphatase & Kinase
gale-1, C47B2.6	0.40	0.11	Metabolism
F36A2.3	0.47	0.16	Metabolism
T27A3.6	0.33	0.26	Metabolism/stress response
Y106G6A.2	0.36	0.17	Protein-protein interactions
C32E12.3, osr-1	0.31	0.06	Stress Response
F08A8.4	0.33	0.14	Metabolism/stress response
T27A3.5	0.31	0.08	Phosphatase & Kinase
T28B8.4	0.31	0.17	Degradation (Ubiquitin & Protease)
Y18D10A.2	0.47	0.16	Degradation (Ubiquitin & Protease)
F55D12.6	0.32	0.09	Protein synthesis/metabolism
F16A11.2	0.37	0.09	Unknown
T08G11.2	0.34	0.07	Signalling/protein-protein
C10G11.6	0.32	0.10	Metabolism
vab-10, ZK1151.1	0.40	0.26	Structural
Y106G6E.4	0.36	0.10	Metabolism
Y65B4BR.5	0.38	0.07	Transcription Factor
Y65B4BR.5	0.50	0.28	Transcription Factor
C41G7.7	0.38	0.18	Unknown
Y53C10A.1	0.52	0.22	Unknown
ZK1225.1	0.45	0.15	Unknown
Y87G2A.5, vrs-2	0.31	0.08	Protein synthesis/metabolism
ZC328.2	0.45	0.05	Transcription Factor
pqn-21, F08B6.5	0.30	0.06	Transcription Factor
C36F7.5	0.32	0.13	Unknown
C41G7.2, klp-16	0.29	0.09	Intracellular Transport
unc-95	0.31	0.16	structural
vps-20, Y65B4A.3			protein sorting / trafficking
tag-138, F08A8.6	0.36	0.15	
tag-138, F08A8.6	0.29	0.08	trafficking
K11D2.4	0.34	0.16	Transcription Factor
no MRC record (likely F59A3.10 by seq)	0.41	0.31	
I-2G20 (no MRC record)	0.34	0.16	
I-2A05 (no MRC record)	0.31	0.08	
I-7O03 (no MRC record)	0.39	0.06	

Gene name	Score (Average)	Standard Deviation	Group
nlp-7, F18E9.2	0.41	0.16	Neuropeptide-Like Protein
C49A9.7	0.38	0.19	Neurokinin receptor
npr-2, T05A1.1	0.38	0.08	neuropeptide receptor
F02E8.2	0.33	0.14	GPCR
C48C5.1	0.29	0.09	GPCR
C16D6.2	0.30	0.17	GPCR

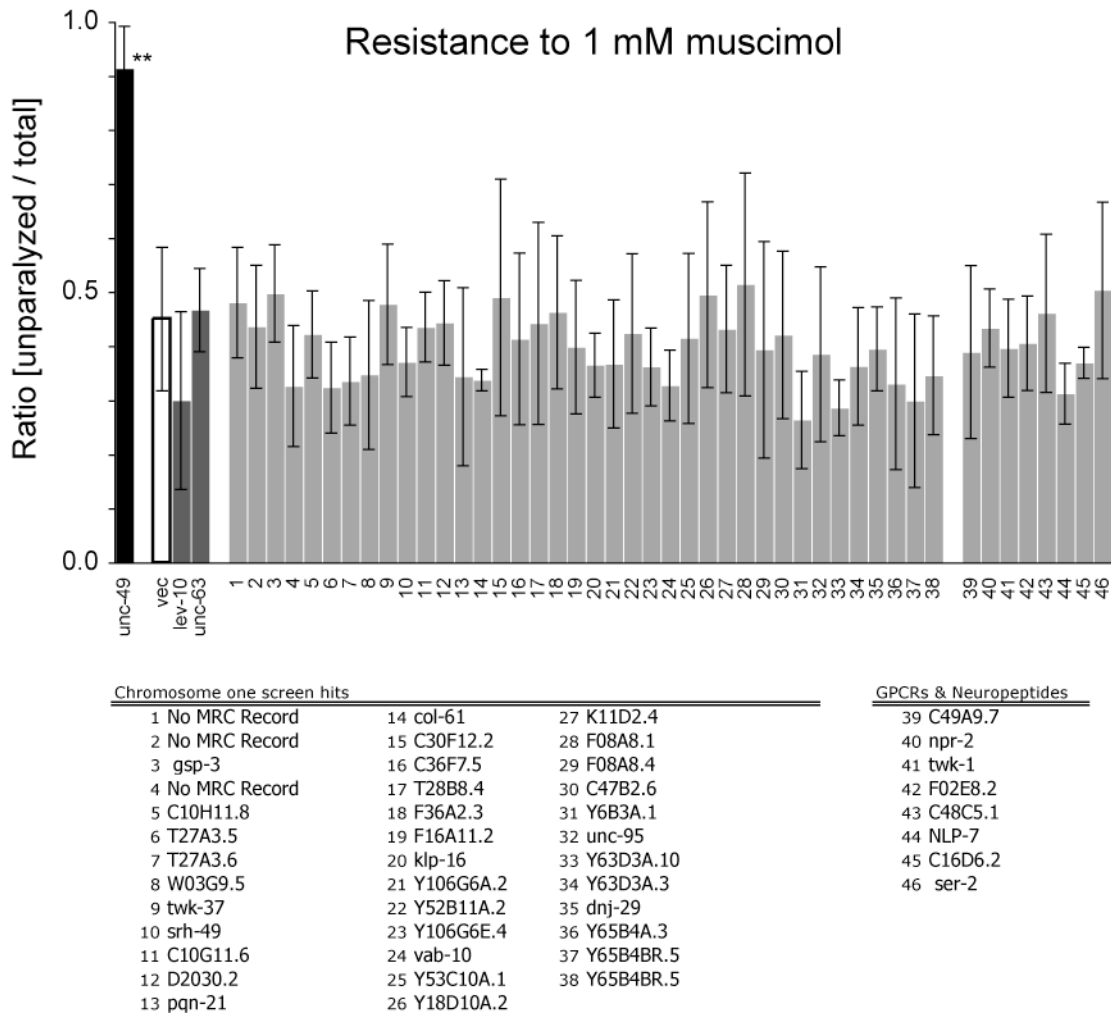


Figure 3.7 Putative Nicotine Resistance Factors not Resistant to Muscimol RNAi treated young adult animals were transferred to 1 mM muscimol NGM plates allowed to incubate for 90 minutes at RT. The number moving (unparalyzed) after mechanical stimulation were counted against the total number of worms observed at 90 minutes. Muscimol is a GABA receptor agonist causing paralysis by hyperpolarizing the muscle, *unc-49* is *C. elegans* only GABA receptor causes resistance to muscimol exposure. [*unc-49*(RNAi) = 91% \pm 3.9 SEM, empty vector = 38% \pm 8.6 SEM, P-value = 0.0066] No other RNAi construct tested demonstrated significant resistance to muscimol. Post-embryonic RNAi induction was used on *rrf-3(pk1426)* for all animals tested.

n = 4, each n represents one trial of > 24 animals, one trials of each animal was run on 3 separate days. Error bars represent the standard deviation among trials. Student t-test, one-tailed, unpaired, significance, * = P < 0.05, ** = P < 0.01, *** = P < 0.001.

CHAPTER IV

Characterization of the Nicotine Resistance Factor *agef-1*

ABSTRACT

Tobacco is one of the most widely used drugs in the world and unquestionably harmful to one's health. The main addictive and psychotropic agent in tobacco is nicotine, which acts through the cholinergic signaling system. Our RNAi based screen of the first chromosome of the *C. elegans* genome unveiled several novel molecules involved in cholinergic signal transmission, a key molecular event in nicotine addiction. One of these genes, *agef-1*, displayed highly significant resistance to nicotine exposure in both our screen and another, similar study, using levamisole—also a cholinergic agonist—by Sieburth *et al.* (2005), here, through pharmacological and behavioral assays, we attempt to further characterize *agef-1*'s role in neurotransmission and transduction of the cholinergic signal in particular. This gene encodes a putative ADP-ribosylation factor's guanine nucleotide exchange factor (Arf-GEF) protein. We demonstrate specificity of *agef-1*'s effect on cholinergic signaling. We go on to demonstrate that AGEF-1 protein is most likely acting post-synaptically; it, however, does not effect the total number of nicotinic acetylcholine receptors at the cell surface. Though, we have localized the cholinergic effect of AGEF-1 to a postsynaptic, intracellular acting protein, our current data are insufficient to provide a model of its activity within the cell.

INTRODUCTION

ADP-ribosylation factors (Arfs) are guanine nucleotide-binding proteins (G-proteins) (Donaldson and Jackson, 2000). Highly conserved members of the Ras superfamily, found in all eukaryotes, Arfs regulate membrane traffic and organelle structure (Li et al., 2004). Activated Arfs bind to intracellular lipid membranes where they recruit lipid-modifying G-proteins and coat complexes involved in trafficking, such as COP1—required for retrograde trafficking between the golgi network and the endoplasmic reticulum. G-proteins are activated by the exchange of guanosine diphosphate (GDP) for the higher energy guanosine triphosphate (GTP), which is slowly hydrolyzed under normal physiological conditions, However this hydrolysis can be speed up by guanine nucleotide exchange factors (GEFs) (Bastiani and Mendel, 2006) The hydrolysis of GTP back to GDP terminates G-protein signaling. Arf-GEFs are a class of GEFs which are specifically involved with the activation of Arf class G-proteins.

Most of the ARF and Arf-GEF research to date has focused on yeast, where the gene classes were discovered, and mammals (Cox et al., 2004). The yeast *Saccharomyces cerevisiae* contains three Arf proteins (arf1 – 3) whereas the human genome encodes six (Arf1 – 6). They contain 5 and 15 Arf-GEFs, respectively. The defining characteristic of an Arf-GEF is the Sec7 domain, which by itself can catalyze the nucleotide exchange on GEF proteins. Mammalian Arfs are heavily involved as signaling molecules which direct the vesicular traffic of the cell and are split into three classes. Class I (Arf1 – 3) is involved in trafficking in the ER-Golgi and endosomal systems, class III (Arf6) is involved in many functions including clathrin endocytosis,

endosomal recycling and actin mediated cytoskeletal movements, whereas little is known about the function of class II Arfs, Arf4 and Arf5 (Donaldson and Jackson, 2000). Yeast *arf-1* and *arf-2* are class I Arfs involved in intercellular transport, endocytosis and exocytosis. They are functionally interchangeable but lethal if both are absent. The third, *arf-3*, is not essential and may play a similar role as mammalian Arf6, a class III Arf. The specificity of Arf-GEFs to Arfs is still an open question in the field, while *in vitro* preferences have been demonstrated, *in vivo* experimentation has shown a single Arf-GEF affecting multiple pathways mediated by distinct Arf proteins (Donaldson and Jackson, 2000).

C. elegans has four identified Arf proteins (*arf-1.1*, *arf-1.2*, *arf-3* and *arf-6*) and five putative Arf-GEFs, identified by sequence homology (*gbf-1*, *efa-6*, *agef-1*, *grp-1* and M02B7.5) (Cox et al., 2004; Li et al., 2004). Not enough work has been done with *C. elegans* Arf proteins to unambiguously determine their classes, as most studies have focused on the mutant and RNAi phenotypes rather than the cell biological classification of the proteins. The *C. elegans* Arfs have been linked to endocytosis and asymmetric cell division, but due to their almost ubiquitous expression coupled with the redundancy shown in other systems, many phenotypes maybe masked in single mutants (Li et al., 2004). Curiously, many of the Arfs have limited mutational effects. However, RNAi depletion often causes severe defects, such as sterility, developmental delays and lethality (Paulini). This might be due to sequence similarity causing knock-down in multiple orthologs. GEFs in *C. elegans*, have been shown to be involved in neurotransmission—including cholinergic

signaling—and rhythmic behaviors—such as locomotion, egg-laying and pharyngeal pumping (feeding) (Norman et al., 2005; Steven et al., 2005).

Previously, we identified an Arf-GEF gene, Y6B3A.1, recently reclassified as *agef-1*, as a potential effector of the cholinergic neurotransmission in an RNAi based screen. RNAi knock-down of *agef-1* consistently showed a resistance to nicotine, a cholinergic agonist. Furthermore, in another RNAi screen, *agef-1* was identified as highly resistant to aldicarb, which inhibits the enzymatic degradation of acetylcholine (ACh) in the synapse, causing an excess of ACh in the synaptic cleft (Sieburth et al., 2005). Both nicotine and aldicarb cause paralysis by hyperstimulating the body wall muscles (BWM) through activation of the nicotinic acetylcholine receptors (nAChRs). Having less functional nAChRs—either competently or numerically—at the cell surface confers resistance to overstimulation by these drugs. Functional nAChRs are pentameric protein complexes that are matured and assembled/oligomerized in the endoplasmic reticulum (ER) in a strictly ordered and controlled manner (Green and Millar, 1995; Smith et al., 1987). Only 30% of the nAChR subunits synthesized are correctly assembled and transported to the cell surface in mouse muscle cells, the rest are catabolized by ER-associated degradation factors (Christianson and Green, 2004; Merlie and Lindstrom, 1983). Furthermore, the malfunction or misexpression of these receptors has been correlated with several pathologies including epilepsy, schizophrenia and nicotine addiction (Newhouse et al., 2004; Ryan, 1999).

Cellular studies in yeast have demonstrated the Arf-GEF class proteins involvement in vesicular trafficking which is critical for nAChRs to be transported

through processing steps in the ER and the golgi and finally to the cell surface for functional use, suggesting a possible function for *agef-1* in expression of functional receptors at the cell surface. Also, a recently identified factor for the trafficking of a subset of matured, assembled nAChRs to the cell surface (UNC-50 in *C. elegans*, UNCL in mice), was demonstrated to bind a yeast Arf-GEF in two-hybrid assays (Eimer et al., 2007; Fitzgerald et al., 2000). Mutants of *unc-50* express fewer nAChRs at the cell surface and are highly resistant to cholinergic agonists, such as nicotine and levamisole (Eimer et al., 2007; Lewis et al., 1980). Here, we investigate the role that this Arf-GEF, *agef-1*, plays in the resistance to nicotine phenotype and its potential role in the expression of functional nAChR at the cell surface.

RESULTS

Identification of agef-1 in RNAi Screens

The *C.elegans* Arf-GEF, *agef-1*, has been identified in two separate RNAi based screens for cholinergic transmission. Seiburth *et al.* identified *agef-1* in a screen searching for gene depletions, which confer aldicarb resistance (2005). Aldicarb is an acetylcholine esterase inhibitor, which prevents the degradation of ACh causing the synaptic cleft to fill with ACh and continuous stimulation of ACh receptors. In the case of the *C. elegans* BWM, this causes uninterrupted contraction resulting in paralysis. RNAi knock-down could confer for resistance to aldicarb in a number of ways: presynaptic ACh release defect, causing less ACh to be released, or

postsynaptic functional defect, if the receptor no longer functioned as effectively or by creating massive structural defects, where the release of ACh is no longer near the corresponding receptors.

We identified *agef-1* in a similar RNAi based screen for nicotine resistance genes. Nicotine is an agonist of the nAChRs, which are main stimulatory receptor on the *C.elegans* BWM. Hyperstimulation of these receptors by nicotine causes simultaneous contraction of the muscles resulting in paralysis—similar to aldicarb, as they both cause over stimulation of the BWMs via the nAChRs. However, because nicotine is an agonist that acts directly on the postsynaptic receptors of the muscle, genes affecting the response to nicotine would be found almost exclusively in the muscle. These results suggest that *agef-1* is a post-synaptic factor involved with the functional expression of nAChRs.

Description of agef-1 and AGEF-1

The *agef-1* gene resides on chromosome one in an operon, CEOP1712, of four other genes, W09C5.1, Y87G2A.11, Y87G2A.13 and *vps-28*, none of which were hits in our nicotine resistance screen and none were tested in the aldicarb based screen (Paulini; Sieburth et al., 2005). It is a predicted Arf-GEF, catalyzing the exchange of GDP for GTP on Arfs activating these G-proteins (Donaldson and Jackson, 2000; Paulini). Three isoforms are predicted for this gene, the longer isoforms, ‘a’ and ‘c,’ are 1594 amino acids (aa) and 1628 aa respectively, and have a Sec7 domain, hallmark of an Arf-GEF protein, from 506 – 709 aa and an Sec7 associated domain of unknown

function (DUF1981) from 1021 – 1105 aa (UniProt, 2010). The Sec7 domain is critically important to the function of Arf-GEFs, it serves at the binding site for GEF molecules and the catalytic site, which dislodges the GDP and allowing GTP to bind and activate the GEF signaling molecule. The shortest isoform, ‘b,’ however, is predicted to be only 551 aa in length, which severely truncates the Sec7 domain, likely impairing, if not abolishing, its ability to activate Arfs. The Sec7 domain defines an Arf-GEF, this isoform would not be considered an Arf-GEF and may act as a non-catalytic competitive inhibitor. Mutation of the *agef-1* gene is homozygous lethal (Paulini). Knock-down by RNAi causes larval lethality in the following generation, making post-embryonic RNAi depletion, as both RNAi screen utilized, one of the few ways to examine this gene (Kamath et al., 2003; Sieburth et al., 2005; Simmer et al., 2003).

agef-1 Expression

Previous microarray studies found *agef-1* to be upregulated in the embryonic motor neurons, as well as three of the other four members of its operon (Fox et al., 2005). However, the screen data also pointed to additional expression in the BWM. We constructed a promoter by amplifying 786 bp upstream of the operon and cloning the PCR product into pD97.95 GFP expression vector (Fire et al.). We found very strong expression in the pharynx—*C. elegans* feeding organ—during all stages of development. We also saw strong expression in the embryo, which was likely pharyngeal expression as well. In adult worms head muscles and BWMs showed slight

expression, but expression was not seen in adult motor neurons. No expression was noted in embryonic motor neurons either, however, due to their small size and the robust expression of GFP in the pharynx, it would be difficult to observe embryonic motor neuron expression.

Cholinergic Agonist Resistance

Our interest in *agef-1* stemmed from its RNAi mediated resistance to the cholinergic agonist, nicotine. Here we show that *agef-1* (RNAi) worms are resistant to both nicotine and levamisole (Fig. 3.2). Unfortunately, *agef-1* is homozygous lethal so we could not assess it for a null mutant phenotype. However, it can be maintained as a heterozygous mutant over a balancer, which should produce less AGEF-1 protein than wild-type. Using these worms we produced worms known to be heterozygous for *agef-1* that could be tested for cholinergic agonist resistances. These heterozygous *agef-1* mutants showed no obvious behavioral defects. These mutants were tested for both nicotine and levamisole resistance and proved slightly statistically resistant to both cholinergic agonists. These heterozygous *agef-1* mutants only showed resistances at long time intervals—the 60 and 80 minute observation times.

Aldicarb and Muscimol Resistance Testing

We sought to further characterize the defects caused by *agef-1* (RNAi). Another screen had identified *agef-1* (RNAi) as strongly resistant to aldicarb (Sieburth

et al., 2005). We recapitulate those finding here (Fig. 3.3.A). The *agef-1* (RNAi) reliably showed significant resistance to alidcarb as previously reported.

Furthermore, to address whether the loss of this molecule gave broad-spectrum drug resistance to worms we tested muscimol. Muscimol is a GABA agonist, which is the other major ionotropic receptor class on the BMWs, when stimulated these cause the influx of anions, hyperpolarizing the cell, which causes paralysis. If *agef-1* were involved universally in receptor maturation of both GABA as well as ACh receptors, or the synapse had collapsed due to massive structural defects causing down regulation of all receptors, this would affect both pathways equally and *agef-1* (RNAi) should confer resistances to both cholinergic and GABAergic agonist (McIntire et al., 1993a). However, if *agef-1* (RNAi) exclusively affects nAChRs' functional expression, then it should not display a muscimol resistance phenotype in addition to its cholinergic resistance phenotype. The *agef-1* (RNAi) worms showed no difference from the wild-type response to muscimol, suggesting it is specifically involved in cholinergic signaling.

Brefeldin A, Arf-GEF Inhibition

Brefeldin A is a fungal metabolite, which stabilizes the Arf-GEF complex with its GEF partner (Donaldson and Jackson, 2000). This complex binds the molecules together without exchanging the GDP for GTP, thus holding the GEF signaling molecule in an inactive state. In yeast and mammalian cell lines, this causes the disassembly of the golgi network and the build-up of immature proteins in the

endoplasmic reticulum. The proteins with the closest sequence similarity to *agef-1* in other organisms (Arf-GEF2 in humans [pblast value = 0]) and YDR170c [pblast value = $6.19e^{-147}$]) have both been demonstrated to be sensitive to brefeldin A (Paulini; Sata et al., 1998). To further characterize the *agef-1* activity we tested if *agef-1* (RNAi) would confer resistance to brefeldin A. No protocols were found to test brefeldin A *in vivo* in *C. elegans*, or other multicellular organisms. Therefore, we attempted to use brefeldin A in a similar manner to the cholinergic agonists and aldicarb, as a movement inhibitor, reasoning that inhibition of *agef-1* would inhibit cholinergic transmission thus retarding locomotion. Unfortunately, our testing with brefeldin A proved to be highly variable as an inhibitor of movement. Our tests indicate that the usable range of 100 – 1000 μ M brefeldin A in NGM plates. We, however, did not observe any statistical difference in the ability of brefeldin A to paralyze *agef-1* (RNAi) and empty vector controls at 100, 500 or 1000 μ M brefeldin A, though high variability among test days causes difficulty in statistical analysis.

As a temporally controlled, potential inhibitor of AGEF-1, we wondered if we could recapitulate the cholinergic resistance in brefeldin A exposed worms. We exposed wild-type worms to 100 - 1000 μ M brefeldin A in NGM plates for half an hour—when they started to exhibit locomotion defects at 1000 μ M—to 2 hours, and then transferred these worms as well as a control group from the original plate to plates with 0.5% nicotine. Movement was assessed every twenty minutes for eighty minutes. No significant difference was found between the any of the brefeldin A treated and non-treated worms.

Effect of agef-1 (RNAi) on the Expression Level of an nAChR Subunit

In attempt to explain the cholinergic resistance observed in agef-1 (RNAi) worms we attempted to quantify the number of nAChRs expressed at the cell-surface. If *agef-1* was involved in the maturation of the nAChR, a depletion of agef-1 by RNAi should reduce the number of nAChR on the post-synaptic NMJ. We had created an epitope tagged nAChR subunit for a previous study, a LEV-1 fusion to hemagglutinin (HA) (Gottschalk and Schafer, 2006). The HA tag is presented extracellularly and can be bound by anti-bodies conjugated to fluorophores injected into the worm's body. The intensity of the light emitted by these fluorophores upon stimulation can be quantified to give a reading of the number of nAChR subunits expressed on the cell surface, as the antibodies cannot pass through the cell's plasma membrane. This technique had proved useful in demonstrating that *unc-50*, an intracellular trafficking protein important to nAChR expression, causes defects in the levels of nAChR expressed at the cell surface (Gottschalk and Schafer). We attempted to show a similar defect in the agef-1 (RNAi) worms by this method. We saw no change in the levels of expression in worms treated with agef-1 (RNAi) from the original worms, lev-10 (RNAi), however, caused a significant drop in the fluorescent intensity (Fig. 4.6).

Dominant Negative to Eliminate AGEF-1 Activity in BWM

Several groups had successfully created dominant negative Arf-GEF proteins in other organisms (Debrececi et al., 2004; Park et al., 1997; Shinotsuka et al., 2002).

These mutated Arf-GEF proteins prevent the exchange of GDP for GTP on the Arf protein and stabilize the interaction between the two molecules, which sequesters the Arf molecule in an inactive state (Beraud-Dufour et al., 1998). In a functional Arf-GEF protein the invariant glutamic acid (E), within the highly conserved motif 'FRLPGE' between the 7th and 8th α -helices of the Sec7 domain, this residue catalyzes the release of the GDP (Cherfils et al., 1998). Dominant negatives are created by mutating this glutamic acid to a lysine (K) within the Arf-GEF defining Sec7 domain. This mutation reduces the exchange rate of GDP to GTP by over four orders of magnitude, in addition to forming an inactive complex with the Arf protein, blocking any functional Arf proteins that maybe present as well (Beraud-Dufour et al., 1998).

We restricted expression of the dominant negative to the muscle cells to assay the loss of the *agef-1* function on the post-synaptic cholinergic receptors, as our data strongly point to post-synaptic activity. We mutated the E to K at amino acid position 607 aa within the Sec7 domain of *agef-1* cDNA and cloned the product into an expression vector under the control of the myo-3 promoter, driving muscle specific expression. By restricting the dominant negative AGEF-1 (E607K) protein expression to the muscles we sought to avoid the lethal effects of complete loss of *agef-1* function. The worms proved viable and had no gross morphological or locomotory defects, like *agef-1* (RNAi). If the *agef-1* was acting post-synaptically in the BWM these transgenic dominant negatives should be resistant to cholinergic agonists. However, our results indicate no significant resistance to nicotine (0.5%) when compared to wild-type worms.

DISCUSSION

These data do not allow a definitive description of the nature of the cholinergic stimulation resistance mediated by *agef-1* (RNAi) observed by our group and others (Sieburth et al., 2005). We have conclusively demonstrated through repeated tests with multiple cholinergic agonists and aldicarb that *agef-1* (RNAi) promotes resistance to cholinergic over stimulation. Likely caused by a defect specifically in cholinergic signaling as muscimol response is unaffected. Previous studies in yeast and mammalian cell-lines have established Arf proteins, and by implication their attendant Arf-GEFs, to be involved with vesicular trafficking, lipid metabolism and microtubule dynamics, also suggesting numerous other cellular processes (Li et al., 2004). This wide array of potential functions does not help us to narrow the possible effects of *agef-1* on cholinergic signaling. Therefore, it was important to establish the cellular location of *agef-1* nicotine resistance to focus our search for potential mechanisms. Our results suggest postsynaptic function as nicotine does not require presynaptic activation to cause paralysis, but we cannot rule out presynaptic effects of development and maintenance of the postsynaptic nAChRs.

A major obstacle in our study was the determination of where the protein was influencing cholinergic signaling, either pre- or postsynaptically. This is a fundamental issue for the investigation of this protein and one that needs to be resolved before further work can establish the molecular mechanisms and pathways.

An unfortunate consequence of RNAi profiling is the inability to rescue phenotypes with a transgenic expression plasmid. Transgenic rescues are the gold standard for localization of a mutant phenotype. Defining a mutant phenotype then expressing the protein in a specific subset of cells to re-create the wild-type response, demonstrates the cells' involvement in that phenotype. However, effective RNAi would degrade both the native and the transgenically expressed gene products. Thus transgenic rescues are not possible to re-construct RNAi induced phenotypes.

Though the expression pattern by promoter fusion to GFP showed very faint expression in the adult BWM we regard these results as somewhat suspect. This expression pattern was generated by the 786 bp region up stream of the operon in which *agef-1* resides. This is also the promoter region for *unc-59*, a cell division protein, a little over 800 bp upstream of the operon. Furthermore, both the operon and the *agef-1* itself have large tracts of intronic regions within them, 55 Kb of the 62 Kb in the operon and 17 Kb of 22 Kb of the gene. These non-coding intronic sequences often alter the expression of the gene in which they reside. Therefore, the large amount of information possibly residing within the introns and the small amount in the promoter make it highly likely that the intronic sequence is significantly modifying the expression pattern of the GFP promoter fusion used here. We thought it prudent to attempt RNAi hybridization to conclusively demonstrate the expression pattern for *agef-1*; however, before this tricky protocol was mastered the decision to terminate this project was made.

Our alternative attempts to answer the question of pre- or postsynaptic site of action proved inconclusive. The expression of a dominant negative *agef-1* *ljEx198* [*Pmyo-3::agef-1 (E607K)*] in the BWM did not affect resistance to nicotine. However, the construct was only a theoretical dominant negative of *agef-1*, designed like similar molecules that had been reported to be dominant negatives (Debreceeni et al., 2004; Park et al., 1997). This construct could not be tested *in vitro* to empirically demonstrate dominant negative activity, because we are unsure of its target molecules and their activities. Also, there has been some evidence that Mg^{++} concentrations higher than 1 mM may inhibit the dominant negative activity of this mutation used (Beraud-Dufour et al., 1998). *In vitro* levels of Mg^{++} above 1 mM have been shown to interfere with the binding of this *Sec7* mutation and its GEF partner. In this instance, the mutated proteins show no ability to catalyze the exchange of GDP for GTP to activate the GEF molecule but it no longer sequesters the GEF to prevent its activation by native GEF proteins and therefore is unlikely to act as a dominant negative. It should be noted, that NGM media on which the animals were grown and test has at least 1 mM of Mg^{++} .

Our test sought to demonstrate *agef-1* function in the BWM. However, we did not look at its effect when expressed in the motor neurons. This was because our results pointed to postsynaptic effects and our interest in the nAChRs specifically, not cholinergic signaling in general. Testing of the dominant negative in the motor neurons could have helped establish the location of our RNAi phenotype.

Attempts to determine if *agef-1* is required for the insertion of nAChRs proved equally inconclusive. The HA tagged nAChR had demonstrated a lower expression of *lev-1* in *unc-50* mutant worms, but was not attempted with *unc-50* (RNAi) (Eimer et al., 2007). It maybe that a knock-down of *agef-1* may not be disruptive enough to cause perturbation of the over expressed tagged nAChR expression. Alternatively, *agef-1* could be involved in the processing of the nAChR sub-units and would not affect the number of nAChRs on the cell surface but their functionality instead. Cholinergic deficiencies causing the phenotypes that we observed could be consistent with either conclusion.

A recently developed method that may address this question is the cell-specific RNAi (Esposito et al., 2007). A method of expressing dsRNA (from two separate transgenic constructs) in a discreet cellular location via *C. elegans* well characterized promoters. Importantly, these effects are localized to the cells expressing the dsRNA, not the systemic response seen by other dsRNA introduction methods. This would allow us to knock-down the expression of *agef-1* in either the muscles or the motor neurons to determine the cells in which this protein acts.

Since the termination of this project, another RNAi based screen identified *agef-1* as highly resistant to the build up of polyglutamine in muscles (Mehta et al., 2009). There are several potential mechanisms for this resistance. One, the loss of the *agef-1*, enhances the activity (proteolysis) of the polyglutamine aggregations, or two, *agef-1* is involved with the build-up of these aberrant polypeptides. Both of these would be consistent with our findings. If the loss of *agef-1* causes hyperactive

degradation, the nAChRs may be prematurely metabolized, leading to fewer nAChRs and mitigating the effects of cholinergic agonists. In the second, *agef-1* is involved with the creation or maturation of these polypeptides. In this event, the nAChRs may not be correctly produced. Another, potential mechanism that would also be consistent with our findings of diminished cholinergic transmission, would be that the cholinergic activity itself is involved with the polyglutamine induced paralysis. If this were the case, the inhibition in the cholinergic transmission caused by the loss of *agef-1*, ultimately slows the progress of the polyglutamine paralysis.

It is interesting to note that in all three studies, the RNAi effect was induced post-embryonically, just after hatch for our study (Sieburth et al 2005, Mehta et al 2009) after the L4 stage of development, the final stage before adulthood in the *C. elegans* lifecycle. Furthermore, we found high levels of embryonic expression and earlier groups noted two generation RNAi treatment was not immediately lethal but caused inviability of the embryos. These observations suggest that *agef-1* is required for development, possibly because *agef-1* is involved in establishing coherent cholinergic signaling—ACh is the only neurotransmitter that is required for survival (Rand, 1989).

MATERIALS & METHODS

Worm Husbandry

The N2 Bristol strain was used as wild-type in this study. *C. elegans* were raised at room temperature (21.5°C) otherwise as describe by Brenner, 1974, on standard nematode growth plates (NGM) agar plates fed with *E. coli* (OP50 strain). All strains, mutants and integrated arrays, were out-crossed a minimum of six times back to the N2 strain before testing.

Young adults were used for all assays. Young adults were staged by placing several worms (3 – 12, depending on desired density and brood size of strain) on *E. coli* seeded NGM plates overnight then removed the following morning (~14 hours). The worms were grown for an additional two days at a room temperature.

Strains used

agef-1 (tm1693) was generously provided by the Japanese National BioResource Project. It was back crossed six times before use in assays. *agef-1 (tm1693)* is a homozygous lethal mutation and was maintained at a heterozygous allele over a *dpy-5* balancer. Transgenic worms expressing HA tagged LEV-1 nAChR subunit, *lev-1 (x427)* lJEx140 [P_{lev-1::lev-1}+HA; rol-6] (AQ598), were created in our lab by Dr. Alexander Gottschalk, and described in Gottschalk & Schafer, 2006, as well as Eimer *et al.* 2007.

Creation of dominant negative AGEF-1 (E607K)

The sequence for *agef-1* was cloned out of a cDNA library, with the JC044 (ggactagtatgagcgaattgtcaaacgggtccgaagaaatcg) and JC045

(ggggtacctcaagtgaaattttccgaaatatggg) primers. This fragment was ligated into a myo-3::YC2.1 backbone, where the YC2.1 had been cut out. Site directed was used to change a guanosine (G) at 1807 bp, the first position of a glutamic acid codon, gaa, to an adenosine (A), which changed the coding to a lysine residue, aaa. This site directed mutagenesis used primers JC056 (ggagaaatttagattaccaggaAAgctcaaaaaatcgatcgctaatggtgaaattcgc) and JC057 (cctctttaaactaatggtcctTTTcgagtttttagctagcggattacaactttaagcg) mutating the underlined nucleoside. This created the construct lJEx198 [Pmyo-3::agef-1(E607K)] which was injected into N2 worms with a Pmyo-2::GFP marker at 100 ng/ μ L and 5 ng/ μ L respectively (Mello et al., 1991).

Pharmacological resistance assays

All plate based resistance assays were done in accordance to the nicotine resistance harsh touch assay as described in Gottschalk, *et al.* 2005 and chapter 3 of this dissertation. The only changes being the drug and the concentration used, as noted in the text of the dissertation.

Liquid based protocols were adapted from the thrashing assay described in Gottschalk, *et al.* 2005. In our assays no ngm agarose was present and movement was scored by eye. The number moving were divided by the total number counted to give a paralysis ratio. The white light source of the dissecting scope was used to stimulate movement. Drugs and concentrations used are noted in the text of this dissertation.

In vivo quantification of nAChR level by anti-body binding

Protocol described in Gottschalk, *et al.* 2005, adapted here to examine the expression level of the nAChR, LEV-1. (Diagramed in figure 4.6.) The parent strain used was *lev-1 (x427) lJEx140 [Plev-1::lev-1+HA; rol-6]* (AQ598), which was injected with anti-HA fluorophore conjugated anti-bodies, 1:400 dilution (clone 16B12, coupled to Alexa488, Molecular Probes). Quantification protocol is also described in Gottschalk, *et al.* 2005.

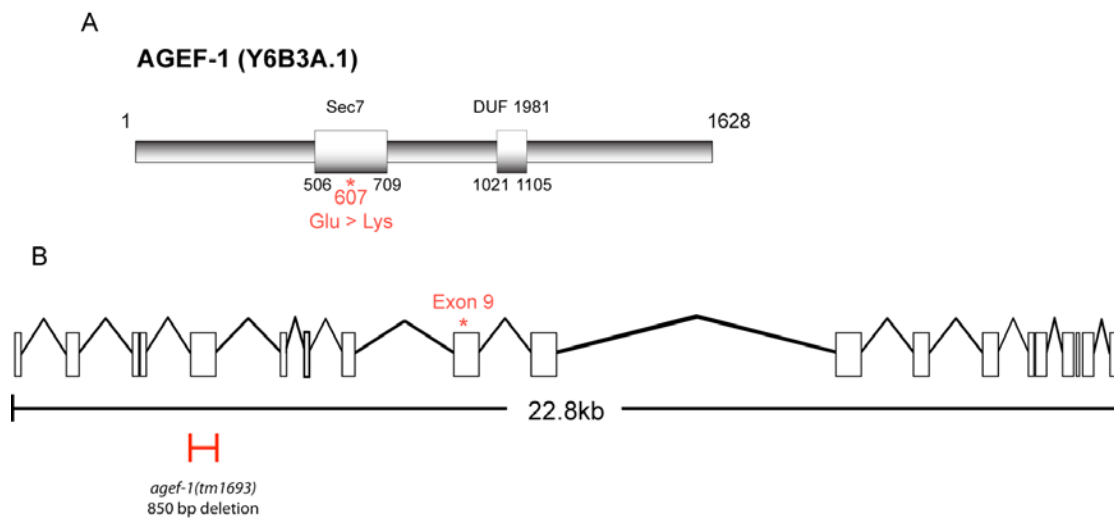
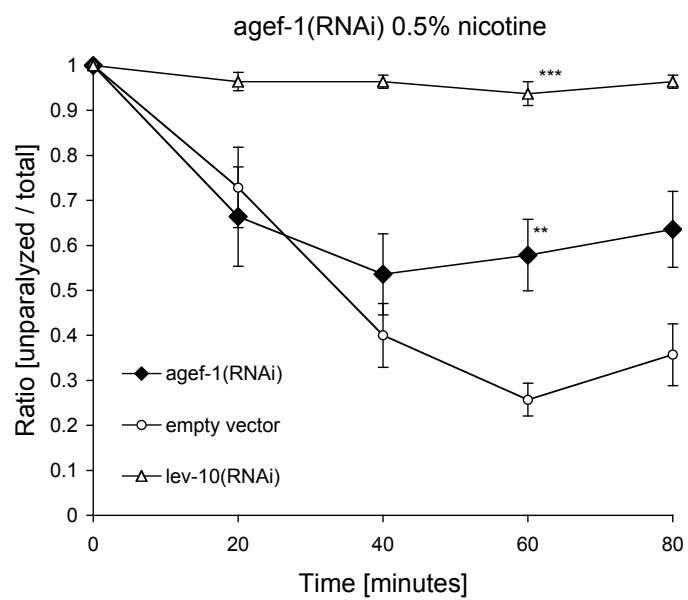


Figure 4.1 *agef-1* Gene and Protein, tm1693 Deletion and E607K Mutation A) AGEF-1 protein showing position Sec7 and DUF 1981 domains. Red star denotes position of glutamic acid to lysine mutation within the Sec7 domain at amino acid 607. B) *agef-1* gene with introns. Red star denotes the location of the E→K mutation at 1807 bp in exon 9. Shows the region deleted in *agef-1 (tm1693)*, the entire exon 5, in addition to a small amount of intronic flanking sequence.

Figure 4.2 agef-1 (RNAi) Confers Resistance to Cholinergic Agonists Young adults treated with post-embryonic RNAi against *agef-1* were transferred to plates with cholinergic agonists [0.5% nicotine or 500 μ M levamisole]. The number moving (unparalyzed) after mechanical stimulation were counted against the total number of worms observed every 20 minutes for 80 minutes. A) Worms treated with *agef-1* (RNAi) are significantly more resistant to 0.5% nicotine paralysis than worms fed empty vector controls. [0.5% nicotine, 60 min., *agef-1* (RNAi) vs. empty vector P = 0.0058] B) *agef-1* (RNAi) worms are similarly more resistant to levamisole as compared to empty vector control worms. [500 μ M levamisole, 60 min., *agef-1* (RNAi) vs. empty vector P = 0.0049]

n = 9, each n represents one trial of > 20 animals, trials were run on at least six separate days. Error bars represent SEM among trials. Significance by Student t-test, one-tailed, unpaired. * = P < 0.05, ** = P < 0.01, *** = P < 0.001. Only the 60-minute time points were considered for statistical analysis and significance.

A



B

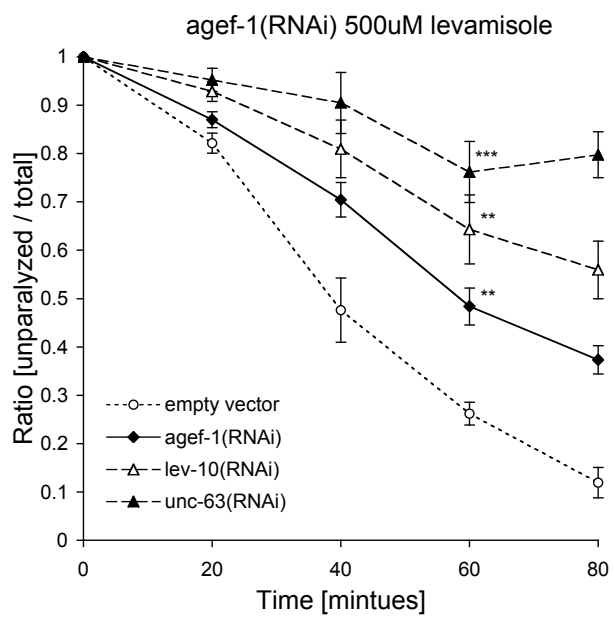
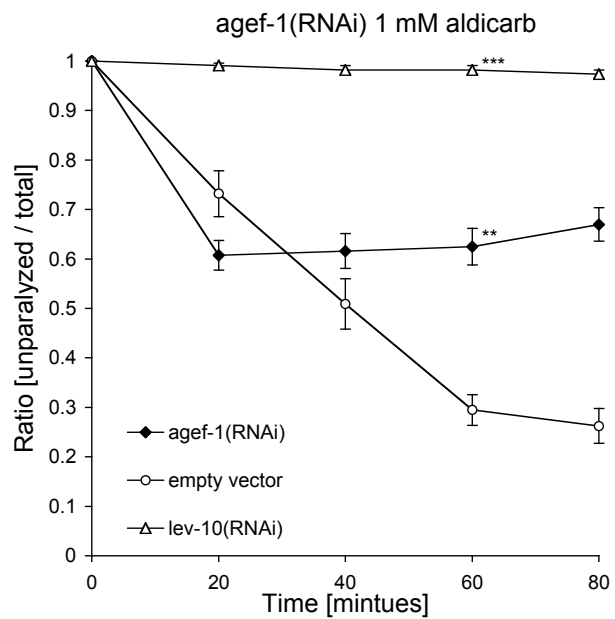


Figure 4.3 agef-1 (RNAi) Confers Aldicarb but not Muscimol Resistance Young adults treated with post-embryonic RNAi were transferred to plates with pharmacological agents [1 mM aldicarb or 1 mM muscimol]. The number moving (unparalyzed) after mechanical stimulation were counted against the total number of worms observed every 20 minutes for 80 minutes for aldicarb and at 90 minutes for muscimol. A) Worms treated with agef-1(RNAi) are significantly more resistant to aldicarb induced paralysis than worms fed empty vector controls. [1 mM aldicarb, 60 min., agef-1 (RNAi) vs. empty vector $P = 0.0088$] B) agef-1 (RNAi) worms were indistinguishable from empty vector controls on 1 mM muscimol. [1 mM muscimol, 60 min., agef-1 (RNAi) vs. empty vector $P = 0.28$]

$n > 12$ for the aldicarb assay where each n represents one trial of > 20 animals, trials were run on at least six separate days. $n = 4$ for the muscimol assay where each n represents one trial of > 30 animals, trials were run on four separate days. Error bars represent SEM among trials. Significance by Student t-test, one-tailed, unpaired. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$. Only the 60-minute time points were considered for statistical analysis and significance.

A



B

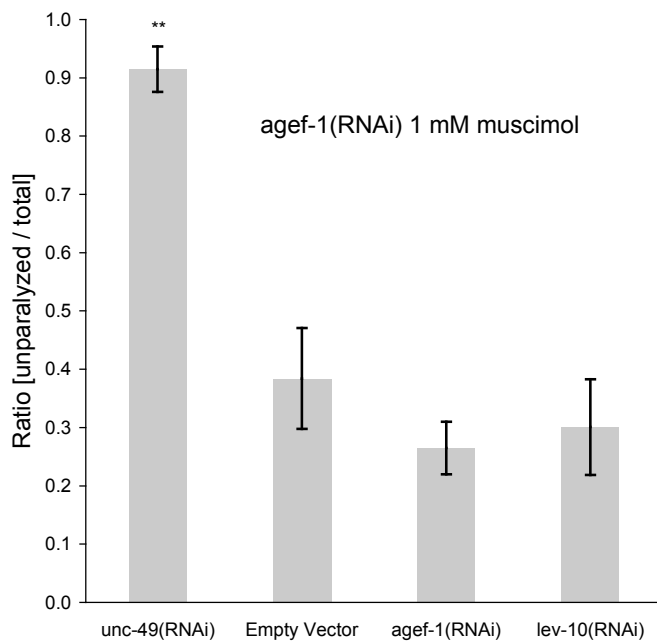
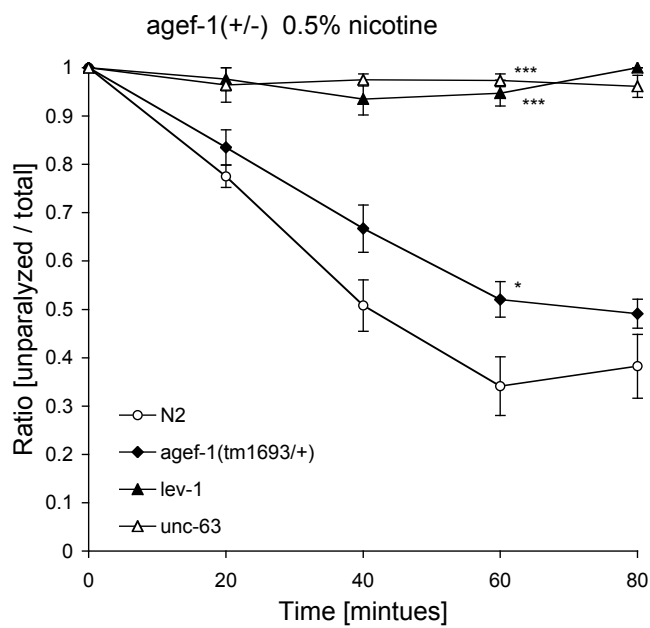


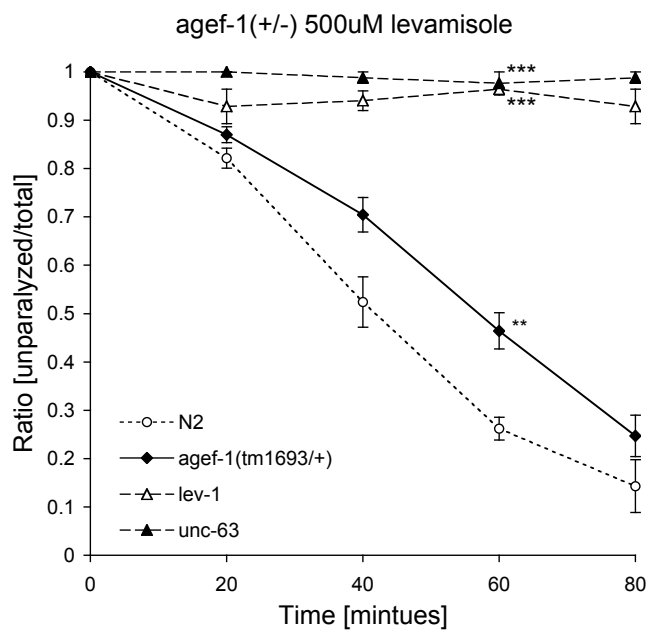
Figure 4.4 Heterozygous *agef-1* Mutation [*agef-1 (tm1693/+)*] Confers Cholinergic Agonist Resistance Young adults heterozygous for *agef-1 (tm1693)* [*agef-1 (tm1693/+)* or *agef-1 (-/+)*] were transferred to plates with cholinergic agonists [0.5% nicotine or 500 μ M levamisole]. The number moving (unparalyzed) after mechanical stimulation were counted against the total number of worms observed every 20 minutes for 80 minutes. A) *agef-1 (tm1693/+)* worms are slightly but significantly more resistant to 0.5% nicotine paralysis than worms fed empty vector controls at 60 min. time point. [0.5% nicotine, 60 min., *agef-1 (tm1693/+)* vs. N2 P = 0.028] B) *agef-1 (tm1693/+)* worms are similarly more resistant to levamisole as compared to empty vector control worms at time point 60. [500 μ M levamisole, 60 min., *agef-1 (tm1693/+)* vs. N2 P = 0.0086]

n = 12 for *agef-1(tm1693/+)*, n = 6 for all other genotypes, each n represents one trial of > 20 animals, trials were run on six separate days. Error bars represent SEM among trials. Significance by Student t-test, one-tailed, unpaired. * = P < 0.05, ** = P < 0.01, *** = P < 0.001. Only the 60-minute time points were considered for statistical analysis and significance.

A



B



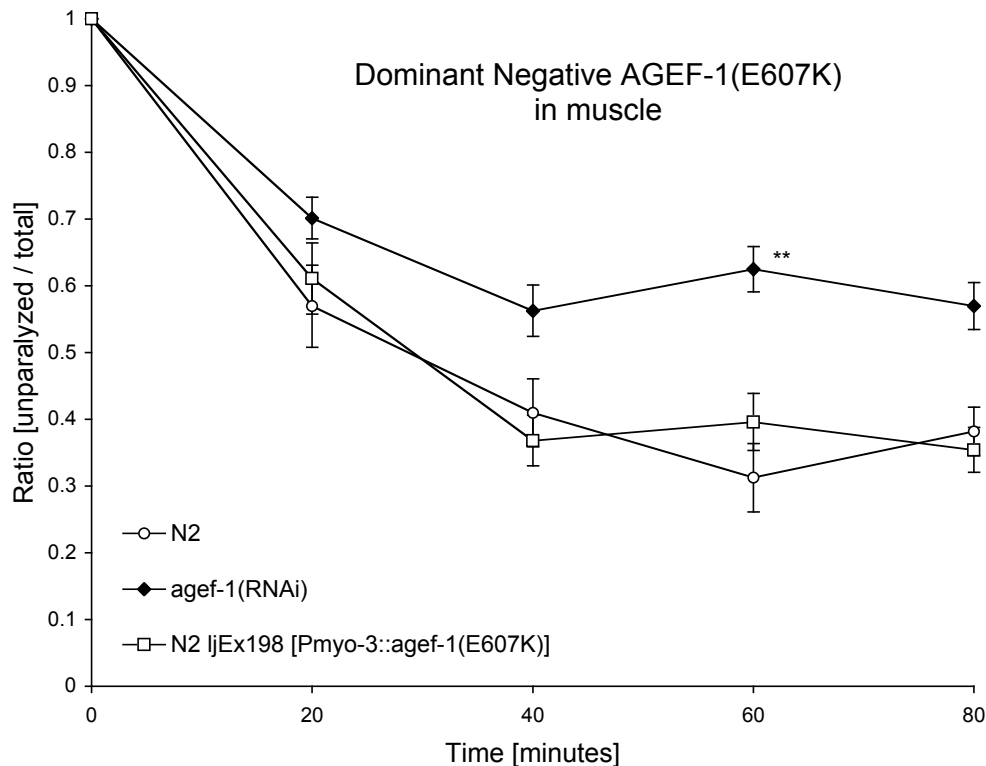
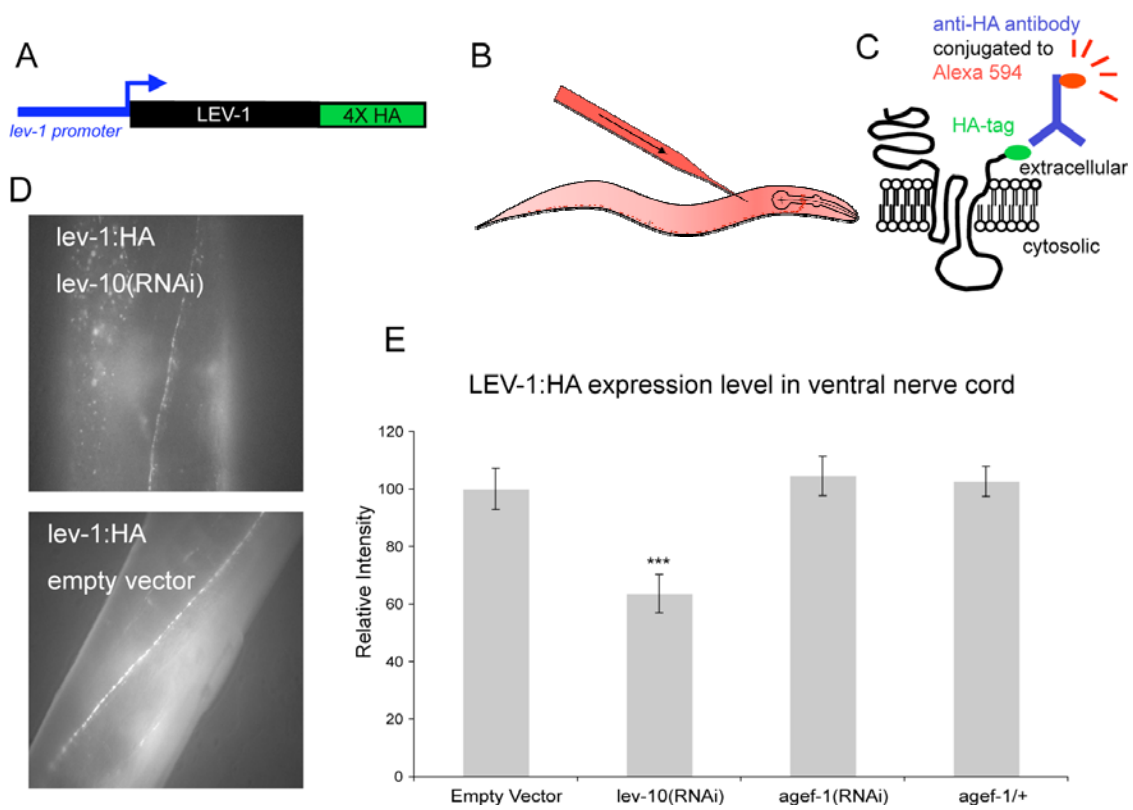


Figure 4.5 Putative Dominant Negative AGEF-1 (E607K) Does Not Confer Resistance to Nicotine Young adults expressing AGEF-1 (E607K) in muscles, *rrf-3* (*pk1426*) treated with post-embryonic *agef-1* (RNAi) and N2s were transferred to plates with 0.5% nicotine. The number moving (unparalyzed) after mechanical stimulation were counted against the total number of worms observed every 20 minutes for 80 minutes. Worms treated with *agef-1* (RNAi) are significantly more resistant to nicotine induced paralysis than N2 worms. [0.5% nicotine, 60 min., *agef-1*(RNAi) vs. N2 $P = 0.0064$], however, the expression of *ljEx198* [Pmyo-3::*agef-1* (E607K)] in the BWM does not confer any resistance to nicotine compared to wild-type. [0.5% nicotine, 60 min., *ljEx198* vs. N2 $P = 0.44$]

$n = 6$, each n represents one trial of > 20 animals, trials were run on three separate days. Error bars represent SEM among trials. Significance by Student t-test, one-tailed, unpaired. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$. Only the 60-minute time points were considered for statistical analysis and significance.

Figure 4.6 Quantification of Surface Expression of an nAChR Subunit in *agef-1* (RNAi) and Heterozygous *agef-1* Mutants Worms expressing an epitope tagged nAChR subunit measure for surface expression by anti-body staining. A) Transgene design for a hemagglutinin (HA) epitope tagged version of *lev-1*. Expression is driven in the BWM by *Plev-1* promoter. B) Anti-bodies raised against HA and conjugated to Alexa-594 fluorophore are injected into the worm's bodily fluids. C) The HA tag is expressed extracellularly to allow for anti-body binding *in vivo* only to nAChR expressed at cell surface. The worms are allowed four to six hours to recover and eliminate unbound anti-bodies. D) Pictures are captured and analyzed for the intensity. This analysis gives a quantification method for the number of nAChRs expressed on the muscle. Intensity values are normalized to an arbitrary units scale with *lev-1 (x427); lJIs4 [Plev-1::lev-1::HA]* serving as wild-type, and being assigned a relative score of 100. E) Wild-type (*lev-1 (x427); lJIs4 [Plev-1::lev-1::HA]* fed empty vector dsRNA expressing bacteria) worms are treated with post-embryonic RNAi and assayed for nAChR surface expression. *lev-10* (RNAi) demonstrates significantly less nAChR expression than wild-type. [empty vector = 100 ± 7.1 SEM, *lev-10* (RNAi) = 63.6 ± 6.6 SEM, P-value = 0.0007]. No statistical difference from empty vector control was found in either *agef-1* (RNAi) [104.5 ± 6.7 SEM, P-value = 0.33] or the *agef-1 (tm1693/+)* crossed into the *lJIs4* expressing background [102.7 ± 5.3 SEM, P-value = 0.39]

n > 20, n represents a single animal, three readings from every animal were averaged for each n. Error bars represent SEM. Significance by Student t-test, one-tailed, unpaired. * = P < 0.05, ** = P < 0.01, *** = P < 0.001.



APPENDIX I

This supplement contains further information on the putative nicotine resistance factors identified in the screen detailed in Chapter III.

Table A.1.1 Further information on putative nicotine resistance factors identified in screening of Chromosome One. (Screen presented in chapter 3 of this thesis.) This table contains the information available about each hit according to wormbase.org. Information was up to date as of January, 2010.

Table A.1.1

Gene name	Function (Putative)	Domain Homology	Expression (Microarray data)	Notes
srh-49, C10G11.4	(olfactory receptor)	7TM GPCR	(upregulated in muscle, implicated in development)	same predicted operon as C10G11.6, also a hit, pnk-1 (lethal involved in aging) and another.
D2030.2	(ATPase)		expressed in intestines	Putative ATP-dependent Clp-type protease (AAA+ ATPase superfamily)
C30F12.2			expressed in neurons, inc. ventral nerve cord, reproductive system, pharynx and hypodermis	AFG1-like ATPase; Sec18p/NSF like domain, priming and fusion of intracellular vesicles
gsp-3, W09C3.6	(calcineurin-like phosphoesterase)			Calcineurin-like phosphoesterase, Metallophosphoesterase, Serine/threonine-specific protein phosphatase
col-61, C01H6.1			expressed in Cuticle (enriched in intestine)	Collagens (type IV and type XIII); Nematode cuticle collagen
hrdl-1, F26E4.11	E3 ubiquitin ligase		(enriched in class A motor neurons)	HRD-like E3 ubiquitin ligase; RING-Zinc finger
fbxb-56, Y63D3A.10	(Protein-Protein interactions)	F-box domain		Cyclin-like F-box; predicted to interact with skp-1p (Yeast) homologs (a core E3 ubiquitin ligase complex component)
C10H11.8	(G-protein signaling)	WD-40 repeats		G-protein beta subunit-like protein (contains WD40 repeats)

Table A.1.1 Continued

Gene name	Function (Putative)	Domain Homology	Expression (Microarray data)	Notes
F36A2.3				Malate dehydrogenase
T27A3.6			enriched in intestine by microarray	Nicotinamide riboside kinase, catalyzes the synthesis of nicotinamide nucleotide (NMN) from nicotinamide riboside; involved in a salvage pathway for NAD+ biosynthesis; mutation of human ortholog leads to disease
Y106G6A.2	(Protein-Protein interactions)	DUF/coiled-coil domain	enriched in muscle and oogenesis by microarray	cDNA confirmed protein; Predicted coiled-coil domain
C32E12.3, osr-1			expressed in intestine and hypodermis; strongly regulated in dauer by microarray	osr-1(rm1) mutant animals maintain normal body volume, motility, and viability even upon chronic exposures to high osmolarity environments; regulates <i>gpdh-1</i>
F08A8.4			(expressed in intestine, expression increases with age)	Acyl-CoA oxidase oxidizes the CoA esters of straight chain fatty acids and prostanolins; increases fat storage
T27A3.5	(Protein tyrosine phosphatase)		(enriched in spermatogenesis)	Protein tyrosine phosphatase; involved in innate immunity response
T28B8.4	(proteasome)		spermatogenesis enriched by microarray	Human mRNA (KIAA0077) product like

Table A.1.1 Continued

Gene name	Function (Putative)	Domain Homology	Expression (Microarray data)	Notes
C10G11.6		SDR		Short-chain dehydrogenase/reductases are one-domain NAD(P)(H)-dependent enzymes with a wide substrate spectrum, ranging from steroids, alcohols, sugars, and aromatic compounds; same predicted operon as srh-49, with gene involved in muscle dev, and pnk-1 involved in ageing
vab-10, ZK1151.1		SH3 & actin binding domains		required for mechanical resilience of the epidermis under strain by the contraction of actin microfilaments mechanically linked to fibrous organelles; RNAi lots of problems, inc unc and egl, actin binding domain, SH3 domain, plectin repeat, Involved in NF-KB signaling; expressed in body wall muscles and many neurons
Y106G6E.4			(enriched in intestines)	5-formyltetrahydrofolate cyclo-ligase, same operon as ced-12 and csnk-1 (casein kinase)

Table A.1.1 Continued

Gene name	Function (Putative)	Domain Homology	Expression (Microarray data)	Notes
ZK1225.1	(involved in development)	DUF domain 1647; 22 proteins in domain family, all in <i>C. elegans</i>		Confirmed protein by cDNA
Y87G2A.5, vrs-2				valyl-tRNA synthetase, a class I aminoacyl-tRNA synthetase that catalyzes the attachment of valine to its cognate tRNA and is thus required for protein biosynthesis; expressed with dauer genes
ZC328.2		Zn-finger, C2H2 type	(upregulated in AWB and AFD [Colosimo et al 2004])	Zn-finger, C2H2 type, involved in axon guidance (Schmitz et al 2007)
pqn-21, F08B6.5		Zn-finger, C2H2 type, nucleic acid binding	expressed in most somatic cells	Prion-like-(Q/N-rich)-domain-bearing protein, involved in learning and memory

Table A.1.1 Continued

Gene name	Function (Putative)	Domain Homology	Expression (Microarray data)	Notes
tag-138, F08A8.6	trafficking	ANTH (4,5 PIP binding) & ENTH (lipid-protein binding)		predicted protein; Huntingtin interacting protein; Actin-binding protein SLA2/Huntingtin-interacting protein Hip1; involved with lipid binding, similar domain to ARFs
K11D2.4	Zn-finger	Zn-finger, C2H2 type, nucleic acid binding		Predicted protein, Zn-finger, C2H2 type
no MRC record--see note				* Likely F59A3.10 by seq/Blast; predicted gene, no domains by homology
RNAi clone: I-2G20 (no MRC record)				
RNAi clone: I-2A05 (no MRC record)				

Table A.1.1 Continued

Gene name	Function (Putative)	Domain Homology	Expression (Microarray data)	Notes
C49A9.7	7TM GPCR, rhodopsin-like, Neurokinin receptor			
npr-2, T05A1.1	7TM GPCR, Serpentine receptor class sx, NPY-like			Enriched in unc-4 expressing cells, mostly cholinergic neurons (Fox et al 2005); depleted in muscles (et al 2005)
F02E8.2	7TM GPCR, rhodopsin-like			Enriched in unc-4 expressing cells, mostly cholinergic neurons (Fox et al 2005)
C48C5.1	7TM GPCR, rhodopsin-like			Enriched in unc-4 expressing cells, mostly cholinergic neurons (Fox et al 2005)
C16D6.2	7TM GPCR, rhodopsin-like, NPY-like			

APPENDIX II

Getting the Most out of an RNAi Screen

In this appendix, I offer some of my own observations as to orchestrating the most successful screen possible. Most of this advice came about from short-comings or missed opportunities I saw in my own screen, presented in chapter 3 of this dissertation. I had intended to write a complete section on designing RNAi screens, however, I found an excellent guide had already been written by Dr. Micheal Boutros and Dr. Julie Ahringer, in *Nature Review Genetics* (2008). Before reading this appendix I would highly recommend review of this article, to which my observations would be a minor supplement.

Run multiple screens/data sets concurrently

In a large scale, high-throughput screen, the major logistical expense is the time and effort it takes to induce the RNAi effect—everything from growing the bacteria up to the plates required. The RNAi treated worms could be used for any screening effort. A lab might consider running an entirely different screens in parallel, or simply trying to glean more information from the testing done. For example, early in the primary screening phase, it was suggested that we look at the nicotine treated worms the next day for defects in adaptation to nicotine. I felt that it was too late to optimize a new component to the screen and this assay was not incorporated into the

screen. I regard this as one of the major missed opportunities of my graduate career. We could have produced a second set of data for what would likely amount to less than an extra hour of work a day—as compared to ~20-30 man hours a day from the four people working on the screen at this time.

More pilot testing

The pilot screen should establish two things; first, it should define the thoroughness and reproducibility of the screen, and second, the ability to logistically manage the scaled up screen. It is needed to ramp up from the primary screen optimization—a few carefully examined assays a day—to full scale screenings as many samples as possible per day. In our screening efforts, the pilot screen only demonstrated the theoretical feasibility of these two factors, but did not rigorously test them empirically. The preliminary testing established a very good primary screening protocol, but the pilot testing was insufficient, which created problems later and made the screening less effective than it could have been.

To end up with a successful screening of the entire genome, or a sub-set of cherry-picked genes, you need to have enough statistical evidence to demonstrate that your screen was ‘saturating.’ With regard to an RNAi screen, you must have confidence that you found all of the genes your screen was capable of finding in the set examined. Our primary screen was not robust enough to claim that we identified all cholinergic resistance genes on chromosome one, rather we have a collections of genes that display a phenotype from chromosome one. (Our hits are valid hits by

RNAi, however to say that we found all the genes on chromosome one which display this phenotype is an over statement.) In hindsight, we should have screened the RNAi treated animals three times in the primary screen for a more robust set of nicotine resistance factors, similar to the protocol followed by Sieburth *et al.* 2005.

Furthermore, we ran into many problems at the beginning of primary testing. Our pilot screen was not run at full capacity, as only two of the four people scheduled for the screen were available. If possible, do the pilot screen as you would your primary screening effort in order to a) find the bottle-necks in the protocol and b) identify any variable that might not have been considered. Ramping up a screen from initial testing or even a pilot screening to a full primary screen will create unforeseen problems, no matter how careful your preparations. The only way to find all of these potential bottle-necks is to run the pilot test at full primary screen numbers. This will also help you determine the number of samples you are able to screen in a day. Second, fully analyze the results of the pilot before the primary screen begins is critical, in the first weeks of the primary screen we found that two of the testers were consistently giving higher scores. This was due to the use of two new microscopes which had the light mounted within the base, heating the base and transferring heat to the worms. This added heat stimulated the worms to move. We did not identify this in the pilot screen, as those particular scopes were not yet in use. The initial testing of the primary screen is to establish a scientifically valid method of search. The pilot screen is not to further test this method at greater volumes (as I had viewed it), but to

establish the logistics necessary for carrying out a robust and thorough examination of every gene in a chosen test set.

More positive controls

Test as many positive controls as possible, in both the initial testing of the primary screening protocol and during the actual screen. We limited ourselves to about half a dozen known cholinergic agonist resistance factors. Understanding which genes display an RNAi phenotype and which do not could be very helpful to your screen, especially if you are planning to examine a targeted set of genes. Also, use more positive controls during the primary screening effort as it is helpful to re-familiarize yourself with your projected target often or you will start to make out spurious observations. Furthermore, if you are using human quantification of your data, recent studies have shown people more readily identify an object or pattern if they are more frequently presented with their positive search criterion (Wolfe and Van Wert, 2010). In conclusion, results would be further optimized by the inclusion of more positive controls, regardless of the screen.

REFERENCES

- (1997). *C. elegans* II. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.
- (2010). The Universal Protein Resource, (ed. T. U. Consortium).
- Ahringer, J.** (2010). Ahringer Lab RNAi webpage, (ed. J. Ahringer).
- Avery, L. and Horvitz, H. R.** (1989). Pharyngeal pumping continues after laser killing of the pharyngeal nervous system of *C. elegans*. *Neuron* **3**, 473-85.
- Bamber, B. A., Beg, A. A., Twyman, R. E. and Jorgensen, E. M.** (1999). The *Caenorhabditis elegans* unc-49 locus encodes multiple subunits of a heteromultimeric GABA receptor. *J Neurosci* **19**, 5348-59.
- Bamber, B. A., Twyman, R. E. and Jorgensen, E. M.** (2003). Pharmacological characterization of the homomeric and heteromeric UNC-49 GABA receptors in *C. elegans*. *Br J Pharmacol* **138**, 883-93.
- Bany, I. A., Dong, M. Q. and Koelle, M. R.** (2003). Genetic and cellular basis for acetylcholine inhibition of *Caenorhabditis elegans* egg-laying behavior. *J Neurosci* **23**, 8060-9.
- Bargmann, C. I.** (1998). Neurobiology of the *Caenorhabditis elegans* genome. *Science* **282**, 2028-33.
- Barnes, N. M. and Sharp, T.** (1999). A review of central 5-HT receptors and their function. *Neuropharmacology* **38**, 1083-152.
- Bastiani, C. and Mendel, J.** (2006). Heterotrimeric G proteins in *C. elegans*. *WormBook*, 1-25.

Beraud-Dufour, S., Robineau, S., Chardin, P., Paris, S., Chabre, M., Cherfils, J. and Antonny, B. (1998). A glutamic finger in the guanine nucleotide exchange factor ARNO displaces Mg²⁺ and the beta-phosphate to destabilize GDP on ARF1. *EMBO J* **17**, 3651-9.

Boutros, M. and Ahringer, J. (2008). The art and design of genetic screens: RNA interference. *Nat Rev Genet* **9**, 554-66.

Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics* **77**, 71-94.

Brodsky, L., Kolotuev, I., Didier, C., Bhoumik, A., Podbilewicz, B. and Ronai, Z. (2004). The LIM domain protein UNC-95 is required for the assembly of muscle attachment structures and is regulated by the RING finger protein RNF-5 in *C. elegans*. *J Cell Biol* **165**, 857-67.

Brose, N., Hofmann, K., Hata, Y. and Sudhof, T. C. (1995). Mammalian homologues of *Caenorhabditis elegans* unc-13 gene define novel family of C2-domain proteins. *J Biol Chem* **270**, 25273-80.

Carnell, L., Illi, J., Hong, S. W. and McIntire, S. L. (2005). The G-protein-coupled serotonin receptor SER-1 regulates egg laying and male mating behaviors in *Caenorhabditis elegans*. *J Neurosci* **25**, 10671-81.

Chalfie, M., White, J. G. (1988). The Nervous System. In *The Nematode Caenorhabditis elegans*, (ed. W. B. Wood). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Chao, M. Y., Komatsu, H., Fukuto, H. S., Dionne, H. M. and Hart, A. C. (2004). Feeding status and serotonin rapidly and reversibly modulate a *Caenorhabditis elegans* chemosensory circuit. *Proc Natl Acad Sci U S A* **101**, 15512-7.

Cherfils, J., Menetrey, J., Mathieu, M., Le Bras, G., Robineau, S., Beraud-Dufour, S., Antonny, B. and Chardin, P. (1998). Structure of the Sec7 domain of the Arf exchange factor ARNO. *Nature* **392**, 101-5.

Christianson, J. C. and Green, W. N. (2004). Regulation of nicotinic receptor expression by the ubiquitin-proteasome system. *EMBO J* **23**, 4156-65.

Cox, R., Mason-Gamer, R. J., Jackson, C. L. and Segev, N. (2004). Phylogenetic analysis of Sec7-domain-containing Arf nucleotide exchangers. *Mol Biol Cell* **15**, 1487-505.

Cronin, C. J., Feng, Z. and Schafer, W. R. (2006). Automated imaging of *C. elegans* behavior. *Methods Mol Biol* **351**, 241-51.

Culetto, E., Baylis, H. A., Richmond, J. E., Jones, A. K., Fleming, J. T., Squire, M. D., Lewis, J. A. and Sattelle, D. B. (2004). The *Caenorhabditis elegans* unc-63 gene encodes a levamisole-sensitive nicotinic acetylcholine receptor alpha subunit. *J Biol Chem* **279**, 42476-83.

Culotti, J. G. and Klein, W. L. (1983). Occurrence of muscarinic acetylcholine receptors in wild type and cholinergic mutants of *Caenorhabditis elegans*. *J Neurosci* **3**, 359-68.

Danaei, G., Ding, E. L., Mozaffarian, D., Taylor, B., Rehm, J., Murray, C. J. and Ezzati, M. (2009). The preventable causes of death in the United States: comparative risk assessment of dietary, lifestyle, and metabolic risk factors. *PLoS Med* **6**, e1000058.

Dasari, S. and Cooper, R. L. (2006). Direct influence of serotonin on the larval heart of *Drosophila melanogaster*. *J Comp Physiol B* **176**, 349-57.

de Bono, M. and Maricq, A. V. (2005). Neuronal substrates of complex behaviors in *C. elegans*. *Annu Rev Neurosci* **28**, 451-501.

de Fiebre, C. M., Meyer, E. M., Henry, J. C., Muraskin, S. I., Kem, W. R. and Papke, R. L. (1995). Characterization of a series of anabaseine-derived compounds reveals that the 3-(4)-dimethylaminocinnamylidene derivative is a selective agonist at neuronal nicotinic alpha 7/125I-alpha-bungarotoxin receptor subtypes. *Mol Pharmacol* **47**, 164-71.

Debreceeni, B., Gao, Y., Guo, F., Zhu, K., Jia, B. and Zheng, Y. (2004). Mechanisms of guanine nucleotide exchange and Rac-mediated signaling revealed by a dominant negative trio mutant. *J Biol Chem* **279**, 3777-86.

Dempsey, C. M., Mackenzie, S. M., Gargus, A., Blanco, G. and Sze, J. Y. (2005). Serotonin (5HT), fluoxetine, imipramine and dopamine target distinct 5HT receptor signaling to modulate *Caenorhabditis elegans* egg-laying behavior. *Genetics* **169**, 1425-36.

Desai, C. and Horvitz, H. R. (1989). *Caenorhabditis elegans* mutants defective in the functioning of the motor neurons responsible for egg laying. *Genetics* **121**, 703-21.

Donaldson, J. G. and Jackson, C. L. (2000). Regulators and effectors of the ARF GTPases. *Curr Opin Cell Biol* **12**, 475-82.

Duerr, J. S., Frisby, D. L., Gaskin, J., Duke, A., Asermely, K., Huddleston, D., Eiden, L. E. and Rand, J. B. (1999). The cat-1 gene of *Caenorhabditis elegans* encodes a vesicular monoamine transporter required for specific monoamine-dependent behaviors. *J Neurosci* **19**, 72-84.

Duerr, J. S., Gaskin, J. and Rand, J. B. (2001). Identified neurons in *C. elegans* coexpress vesicular transporters for acetylcholine and monoamines. *Am J Physiol Cell Physiol* **280**, C1616-22.

Eimer, S., Gottschalk, A., Hengartner, M., Horvitz, H. R., Richmond, J., Schafer, W. R. and Bessereau, J. L. (2007). Regulation of nicotinic receptor trafficking by the transmembrane Golgi protein UNC-50. *EMBO J* **26**, 4313-23.

Erickson, J. D., Varoqui, H., Schafer, M. K., Modi, W., Diebler, M. F., Weihe, E., Rand, J., Eiden, L. E., Bonner, T. I. and Usdin, T. B. (1994). Functional identification of a vesicular acetylcholine transporter and its expression from a "cholinergic" gene locus. *J Biol Chem* **269**, 21929-32.

Esposito, G., Di Schiavi, E., Bergamasco, C. and Bazzicalupo, P. (2007). Efficient and cell specific knock-down of gene function in targeted *C. elegans* neurons. *Gene* **395**, 170-6.

Fire, A., Harrison, S. W. and Dixon, D. (1990). A modular set of lacZ fusion vectors for studying gene expression in *Caenorhabditis elegans*. *Gene* **93**, 189-98.

Fitzgerald, J., Kennedy, D., Viseshakul, N., Cohen, B. N., Mattick, J., Bateman, J. F. and Forsayeth, J. R. (2000). UNCL, the mammalian homologue of UNC-50, is an inner nuclear membrane RNA-binding protein. *Brain Res* **877**, 110-23.

Fleming, J. T., Squire, M. D., Barnes, T. M., Tornoe, C., Matsuda, K., Ahnn, J., Fire, A., Sulston, J. E., Barnard, E. A., Sattelle, D. B. et al. (1997). *Caenorhabditis elegans* levamisole resistance genes *lev-1*, *unc-29*, and *unc-38* encode functional nicotinic acetylcholine receptor subunits. *J Neurosci* **17**, 5843-57.

Forrester, W. C., Kim, C. and Garriga, G. (2004). The *Caenorhabditis elegans* Ror RTK CAM-1 inhibits EGL-20/Wnt signaling in cell migration. *Genetics* **168**, 1951-62.

Fox, R. M., Von Stetina, S. E., Barlow, S. J., Shaffer, C., Olszewski, K. L., Moore, J. H., Dupuy, D., Vidal, M. and Miller, D. M., 3rd. (2005). A gene expression fingerprint of *C. elegans* embryonic motor neurons. *BMC Genomics* **6**, 42.

Francis, M. M., Evans, S. P., Jensen, M., Madsen, D. M., Mancuso, J., Norman, K. R. and Maricq, A. V. (2005). The Ror receptor tyrosine kinase CAM-1 is required for ACR-16-mediated synaptic transmission at the *C. elegans* neuromuscular junction. *Neuron* **46**, 581-94.

Fraser, A. G., Kamath, R. S., Zipperlen, P., Martinez-Campos, M., Sohrmann, M. and Ahringer, J. (2000). Functional genomic analysis of *C. elegans* chromosome I by systematic RNA interference. *Nature* **408**, 325-30.

Furshpan, E. J., MacLeish, P. R., O'Lague, P. H. and Potter, D. D. (1976). Chemical transmission between rat sympathetic neurons and cardiac myocytes developing in microcultures: evidence for cholinergic, adrenergic, and dual-function neurons. *Proc Natl Acad Sci U S A* **73**, 4225-9.

Gally, C., Eimer, S., Richmond, J. E. and Bessereau, J. L. (2004). A transmembrane protein required for acetylcholine receptor clustering in *Caenorhabditis elegans*. *Nature* **431**, 578-82.

Gottschalk, A. and Schafer, W. R. (2006). Visualization of integral and peripheral cell surface proteins in live *Caenorhabditis elegans*. *J Neurosci Methods* **154**, 68-79.

Green, W. N. and Millar, N. S. (1995). Ion-channel assembly. *Trends Neurosci* **18**, 280-7.

Grishok, A. and Mello, C. C. (2002). RNAi (Nematodes: *Caenorhabditis elegans*). *Adv Genet* **46**, 339-60.

Hajdu-Cronin, Y. M., Chen, W. J., Patikoglou, G., Koelle, M. R. and Sternberg, P. W. (1999). Antagonism between G(o)alpha and G(q)alpha in *Caenorhabditis elegans*: the RGS protein EAT-16 is necessary for G(o)alpha signaling and regulates G(q)alpha activity. *Genes Dev* **13**, 1780-93.

Halevi, S., Yassin, L., Eshel, M., Sala, F., Sala, S., Criado, M. and Treinin, M. (2003). Conservation within the RIC-3 gene family. Effectors of mammalian nicotinic acetylcholine receptor expression. *J Biol Chem* **278**, 34411-7.

Hannon, J. and Hoyer, D. (2008). Molecular biology of 5-HT receptors. *Behav Brain Res* **195**, 198-213.

Hapiak, V. M., Hobson, R. J., Hughes, L., Smith, K., Harris, G., Condon, C., Komuniecki, P. and Komuniecki, R. W. (2009). Dual excitatory and inhibitory serotonergic inputs modulate egg laying in *Caenorhabditis elegans*. *Genetics* **181**, 153-63.

Hardaker, L. A., Singer, E., Kerr, R., Zhou, G. and Schafer, W. R. (2001). Serotonin modulates locomotory behavior and coordinates egg-laying and movement in *Caenorhabditis elegans*. *J Neurobiol* **49**, 303-13.

Harfe, B. D. and Fire, A. (1998). Muscle and nerve-specific regulation of a novel NK-2 class homeodomain factor in *Caenorhabditis elegans*. *Development* **125**, 421-9.

Harris, G. P., Hapiak, V. M., Wragg, R. T., Miller, S. B., Hughes, L. J., Hobson, R. J., Steven, R., Bamber, B. and Komuniecki, R. W. (2009). Three distinct amine receptors operating at different levels within the locomotory circuit are each essential for the serotonergic modulation of chemosensation in *Caenorhabditis elegans*. *J Neurosci* **29**, 1446-56.

- Hobson, R. J., Geng, J., Gray, A. D. and Komuniecki, R. W.** (2003). SER-7b, a constitutively active Galphas coupled 5-HT7-like receptor expressed in the *Caenorhabditis elegans* M4 pharyngeal motorneuron. *J Neurochem* **87**, 22-9.
- Hobson, R. J., Hapiak, V. M., Xiao, H., Buehrer, K. L., Komuniecki, P. R. and Komuniecki, R. W.** (2006). SER-7, a *Caenorhabditis elegans* 5-HT7-like receptor, is essential for the 5-HT stimulation of pharyngeal pumping and egg laying. *Genetics* **172**, 159-69.
- Horvitz, H. R., Chalfie, M., Trent, C., Sulston, J. E. and Evans, P. D.** (1982). Serotonin and octopamine in the nematode *Caenorhabditis elegans*. *Science* **216**, 1012-4.
- Hwang, J. M., Chang, D. J., Kim, U. S., Lee, Y. S., Park, Y. S., Kaang, B. K. and Cho, N. J.** (1999). Cloning and functional characterization of a *Caenorhabditis elegans* muscarinic acetylcholine receptor. *Receptors Channels* **6**, 415-24.
- Jansen, G., Thijssen, K. L., Werner, P., van der Horst, M., Hazendonk, E. and Plasterk, R. H.** (1999). The complete family of genes encoding G proteins of *Caenorhabditis elegans*. *Nat Genet* **21**, 414-9.
- Jones, A. K. and Sattelle, D. B.** (2004). Functional genomics of the nicotinic acetylcholine receptor gene family of the nematode, *Caenorhabditis elegans*. *Bioessays* **26**, 39-49.
- Jose, A. M., Bany, I. A., Chase, D. L. and Koelle, M. R.** (2007). A specific subset of transient receptor potential vanilloid-type channel subunits in *Caenorhabditis elegans* endocrine cells function as mixed heteromers to promote neurotransmitter release. *Genetics* **175**, 93-105.
- Kamath, R. S. and Ahringer, J.** (2003). Genome-wide RNAi screening in *Caenorhabditis elegans*. *Methods* **30**, 313-21.
- Kamath, R. S., Fraser, A. G., Dong, Y., Poulin, G., Durbin, R., Gotta, M., Kanapin, A., Le Bot, N., Moreno, S., Sohrmann, M. et al.** (2003). Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. *Nature* **421**, 231-7.

Kamath, R. S., Martinez-Campos, M., Zipperlen, P., Fraser, A. G. and Ahringer, J. (2001). Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in *Caenorhabditis elegans*. *Genome Biol* **2**, RESEARCH0002.

Kandel E.R., S. J. H., Jessell T.M., Ed. (2000). Principles of Neural Science. New York: McGraw-Hill.

Kerr, R., Lev-Ram, V., Baird, G., Vincent, P., Tsien, R. Y. and Schafer, W. R. (2000). Optical imaging of calcium transients in neurons and pharyngeal muscle of *C. elegans*. *Neuron* **26**, 583-94.

Kerr, R. A. and Schafer, W. R. (2006). Intracellular Ca²⁺ imaging in *C. elegans*. *Methods Mol Biol* **351**, 253-64.

Kihara, T., Shimohama, S., Sawada, H., Honda, K., Nakamizo, T., Shibasaki, H., Kume, T. and Akaike, A. (2001). alpha 7 nicotinic receptor transduces signals to phosphatidylinositol 3-kinase to block A beta-amyloid-induced neurotoxicity. *J Biol Chem* **276**, 13541-6.

Kim, J., Poole, D. S., Waggoner, L. E., Kempf, A., Ramirez, D. S., Treschow, P. A. and Schafer, W. R. (2001). Genes affecting the activity of nicotinic receptors involved in *Caenorhabditis elegans* egg-laying behavior. *Genetics* **157**, 1599-610.

Kim, K. and Li, C. (2004). Expression and regulation of an FMR1-related neuropeptide gene family in *Caenorhabditis elegans*. *J Comp Neurol* **475**, 540-50.

Kluger, R. (1996). Ashes to Ashes: America's Hundred-Year Cigarette War, the Public Health, and the Unbashed Triumph of Philip Morris: Knopf.

Komuniecki, R. W., Hobson, R. J., Rex, E. B., Hapiak, V. M. and Komuniecki, P. R. (2004). Biogenic amine receptors in parasitic nematodes: what can be learned from *Caenorhabditis elegans*? *Mol Biochem Parasitol* **137**, 1-11.

Laviolette, S. R. and van der Kooy, D. (2004). The neurobiology of nicotine addiction: bridging the gap from molecules to behaviour. *Nat Rev Neurosci* **5**, 55-65.

Lee, Y. S., Park, Y. S., Chang, D. J., Hwang, J. M., Min, C. K., Kaang, B. K. and Cho, N. J. (1999). Cloning and expression of a G protein-linked acetylcholine receptor from *Caenorhabditis elegans*. *J Neurochem* **72**, 58-65.

Lee, Y. S., Park, Y. S., Nam, S., Suh, S. J., Lee, J., Kaang, B. K. and Cho, N. J. (2000). Characterization of GAR-2, a novel G protein-linked acetylcholine receptor from *Caenorhabditis elegans*. *J Neurochem* **75**, 1800-9.

Leonard, S. and Bertrand, D. (2001). Neuronal nicotinic receptors: from structure to function. *Nicotine Tob Res* **3**, 203-23.

Lewis, J. A., Elmer, J. S., Skimming, J., McLafferty, S., Fleming, J. and McGee, T. (1987a). Cholinergic receptor mutants of the nematode *Caenorhabditis elegans*. *J Neurosci* **7**, 3059-71.

Lewis, J. A., Fleming, J. T., McLafferty, S., Murphy, H. and Wu, C. (1987b). The levamisole receptor, a cholinergic receptor of the nematode *Caenorhabditis elegans*. *Mol Pharmacol* **31**, 185-93.

Lewis, J. A., Wu, C. H., Levine, J. H. and Berg, H. (1980). Levamisole-resistant mutants of the nematode *Caenorhabditis elegans* appear to lack pharmacological acetylcholine receptors. *Neuroscience* **5**, 967-89.

Li, Y., Kelly, W. G., Logsdon, J. M., Jr., Schurko, A. M., Harfe, B. D., Hill-Harfe, K. L. and Kahn, R. A. (2004). Functional genomic analysis of the ADP-ribosylation factor family of GTPases: phylogeny among diverse eukaryotes and function in *C. elegans*. *FASEB J* **18**, 1834-50.

Luther, J. A. B., S.J. (2009). Co-release of norepinephrine and acetylcholine by mammalian sympathetic neurons: regulation by target-derived signaling. In *Co-existence and co-release of classical neurotransmitters*, (ed. R. Gutierrez), pp. 35 - 53: Springer-Verlag.

McIntire, S. L., Jorgensen, E. and Horvitz, H. R. (1993a). Genes required for GABA function in *Caenorhabditis elegans*. *Nature* **364**, 334-7.

- McIntire, S. L., Jorgensen, E., Kaplan, J. and Horvitz, H. R.** (1993b). The GABAergic nervous system of *Caenorhabditis elegans*. *Nature* **364**, 337-41.
- Mehta, R., Steinkraus, K. A., Sutphin, G. L., Ramos, F. J., Shamieh, L. S., Huh, A., Davis, C., Chandler-Brown, D. and Kaeberlein, M.** (2009). Proteasomal regulation of the hypoxic response modulates aging in *C. elegans*. *Science* **324**, 1196-8.
- Mello, C. and Fire, A.** (1995). DNA transformation. *Methods Cell Biol* **48**, 451-82.
- Mello, C. C., Kramer, J. M., Stinchcomb, D. and Ambros, V.** (1991). Efficient gene transfer in *C. elegans*: extrachromosomal maintenance and integration of transforming sequences. *EMBO J* **10**, 3959-70.
- Merlie, J. P. and Lindstrom, J.** (1983). Assembly in vivo of mouse muscle acetylcholine receptor: identification of an alpha subunit species that may be an assembly intermediate. *Cell* **34**, 747-57.
- Miller, K. G., Emerson, M. D. and Rand, J. B.** (1999). Galpha and diacylglycerol kinase negatively regulate the Gqalpha pathway in *C. elegans*. *Neuron* **24**, 323-33.
- Miller, K. G. and Rand, J. B.** (2000). A role for RIC-8 (Synembryn) and GOA-1 (G(o)alpha) in regulating a subset of centrosome movements during early embryogenesis in *Caenorhabditis elegans*. *Genetics* **156**, 1649-60.
- Miyawaki, A., Griesbeck, O., Heim, R. and Tsien, R. Y.** (1999). Dynamic and quantitative Ca²⁺ measurements using improved cameleons. *Proc Natl Acad Sci U S A* **96**, 2135-40.
- Nathoo, A. N., Moeller, R. A., Westlund, B. A. and Hart, A. C.** (2001). Identification of neuropeptide-like protein gene families in *Caenorhabditis elegans* and other species. *Proc Natl Acad Sci U S A* **98**, 14000-5.
- Newhouse, P., Singh, A. and Potter, A.** (2004). Nicotine and nicotinic receptor involvement in neuropsychiatric disorders. *Curr Top Med Chem* **4**, 267-82.

Newhouse, P. A., Potter, A., Kelton, M. and Corwin, J. (2001). Nicotinic treatment of Alzheimer's disease. *Biol Psychiatry* **49**, 268-78.

Norman, K. R., Fazio, R. T., Mellem, J. E., Espelt, M. V., Strange, K., Beckerle, M. C. and Maricq, A. V. (2005). The Rho/Rac-family guanine nucleotide exchange factor VAV-1 regulates rhythmic behaviors in *C. elegans*. *Cell* **123**, 119-32.

Nurrish, S., Segalat, L. and Kaplan, J. M. (1999). Serotonin inhibition of synaptic transmission: Galpha(0) decreases the abundance of UNC-13 at release sites. *Neuron* **24**, 231-42.

Olde, B. and McCombie, W. R. (1997). Molecular cloning and functional expression of a serotonin receptor from *Caenorhabditis elegans*. *J Mol Neurosci* **8**, 53-62.

Opperman, C. H. and Chang, S. (1991). Effects of Aldicarb and Fenamiphos on Acetylcholinesterase and Motility of *Caenorhabditis elegans*. *J Nematol* **23**, 20-7.

Park, W., Mosteller, R. D. and Broek, D. (1997). Identification of a dominant-negative mutation in the yeast CDC25 guanine nucleotide exchange factor for Ras. *Oncogene* **14**, 831-6.

Paulini, M. (2010). wormbase.org, vol. 2010 (ed. P. Strenberg).

Putrenko, I., Zakikhani, M. and Dent, J. A. (2005). A family of acetylcholine-gated chloride channel subunits in *Caenorhabditis elegans*. *J Biol Chem* **280**, 6392-8.

Rand, J. B. (1989). Genetic analysis of the cha-1-unc-17 gene complex in *Caenorhabditis*. *Genetics* **122**, 73-80.

Rand, J. B. (2007). Acetylcholine. *WormBook*, 1-21.

Rand, J. B. and Russell, R. L. (1985). Molecular basis of drug-resistance mutations in *C. elegans*. *Psychopharmacol Bull* **21**, 623-30.

Ranganathan, R., Cannon, S. C. and Horvitz, H. R. (2000). MOD-1 is a serotonin-gated chloride channel that modulates locomotory behaviour in *C. elegans*. *Nature* **408**, 470-5.

Rashid, M. H. and Ueda, H. (2002). Neuropathy-specific analgesic action of intrathecal nicotinic agonists and its spinal GABA-mediated mechanism. *Brain Res* **953**, 53-62.

Reigl, M., Alon, U. and Chklovskii, D. B. (2004). Search for computational modules in the *C. elegans* brain. *BMC Biol* **2**, 25.

Richmond, J. E. and Jorgensen, E. M. (1999). One GABA and two acetylcholine receptors function at the *C. elegans* neuromuscular junction. *Nat Neurosci* **2**, 791-7.

Ringstad, N. and Horvitz, H. R. (2008). FMRamide neuropeptides and acetylcholine synergistically inhibit egg-laying by *C. elegans*. *Nat Neurosci* **11**, 1168-76.

Roth B.L., L. E., Patel S., Wesley W.K. (2000). The Multiplicity of Serotonin Receptors: Uselessly Diverse Molecules or an Embarrassment of Riches? *The Neuroscientist* **6**, 252 - 262.

Rual, J. F., Ceron, J., Koreth, J., Hao, T., Nicot, A. S., Hirozane-Kishikawa, T., Vandenhaute, J., Orkin, S. H., Hill, D. E., van den Heuvel, S. et al. (2004). Toward improving *Caenorhabditis elegans* phenome mapping with an ORFeome-based RNAi library. *Genome Res* **14**, 2162-8.

Ryan, S. G. (1999). Ion channels and the genetic contribution to epilepsy. *J Child Neurol* **14**, 58-66.

Sanes, J. R. and Lichtman, J. W. (2001). Induction, assembly, maturation and maintenance of a postsynaptic apparatus. *Nat Rev Neurosci* **2**, 791-805.

Sata, M., Donaldson, J. G., Moss, J. and Vaughan, M. (1998). Brefeldin A-inhibited guanine nucleotide-exchange activity of Sec7 domain from yeast Sec7 with yeast and mammalian ADP ribosylation factors. *Proc Natl Acad Sci U S A* **95**, 4204-8.

Schafer, W. F. (2006). Genetics of egg-laying in worms. *Annu Rev Genet* **40**, 487-509.

Schafer, W. R. and Kenyon, C. J. (1995). A calcium-channel homologue required for adaptation to dopamine and serotonin in *Caenorhabditis elegans*. *Nature* **375**, 73-8.

Schafer, W. R., Sanchez, B. M. and Kenyon, C. J. (1996). Genes affecting sensitivity to serotonin in *Caenorhabditis elegans*. *Genetics* **143**, 1219-30.

Schinkmann, K. and Li, C. (1992). Localization of FMRFamide-like peptides in *Caenorhabditis elegans*. *J Comp Neurol* **316**, 251-60.

Schmitz, C., Kinge, P. and Hutter, H. (2007). Axon guidance genes identified in a large-scale RNAi screen using the RNAi-hypersensitive *Caenorhabditis elegans* strain nre-1(hd20) lin-15b(hd126). *Proc Natl Acad Sci U S A* **104**, 834-9.

Schutz, B., von Engelhardt, J., Gordes, M., Schafer, M. K., Eiden, L. E., Monyer, H. and Weihe, E. (2008). Sweat gland innervation is pioneered by sympathetic neurons expressing a cholinergic/noradrenergic co-phenotype in the mouse. *Neuroscience* **156**, 310-8.

Shinotsuka, C., Waguri, S., Wakasugi, M., Uchiyama, Y. and Nakayama, K. (2002). Dominant-negative mutant of BIG2, an ARF-guanine nucleotide exchange factor, specifically affects membrane trafficking from the trans-Golgi network through inhibiting membrane association of AP-1 and GGA coat proteins. *Biochem Biophys Res Commun* **294**, 254-60.

Shyn, S. I. (2003). Cameleon Reveals a Physiologic Correlate for Alternative Behavioral States in *Caenorhabditis elegans* Egg-laying. In *Biology*, vol. Ph.D. (ed., pp. 119. San Diego: University of California San Diego.

Shyn, S. I., Kerr, R. and Schafer, W. R. (2003). Serotonin and Go modulate functional states of neurons and muscles controlling *C. elegans* egg-laying behavior. *Curr Biol* **13**, 1910-5.

Sieburth, D., Ch'ng, Q., Dybbs, M., Tavazoie, M., Kennedy, S., Wang, D., Dupuy, D., Rual, J. F., Hill, D. E., Vidal, M. et al. (2005). Systematic analysis of genes required for synapse structure and function. *Nature* **436**, 510-7.

Simmer, F., Moorman, C., van der Linden, A. M., Kuijk, E., van den Berghe, P. V., Kamath, R. S., Fraser, A. G., Ahringer, J. and Plasterk, R. H. (2003). Genome-wide RNAi of *C. elegans* using the hypersensitive rrf-3 strain reveals novel gene functions. *PLoS Biol* **1**, E12.

Simmer, F., Tijsterman, M., Parrish, S., Koushika, S. P., Nonet, M. L., Fire, A., Ahringer, J. and Plasterk, R. H. (2002). Loss of the putative RNA-directed RNA polymerase RRF-3 makes *C. elegans* hypersensitive to RNAi. *Curr Biol* **12**, 1317-9.

Smith, M. M., Lindstrom, J. and Merlie, J. P. (1987). Formation of the alpha-bungarotoxin binding site and assembly of the nicotinic acetylcholine receptor subunits occur in the endoplasmic reticulum. *J Biol Chem* **262**, 4367-76.

Steven, R., Zhang, L., Culotti, J. and Pawson, T. (2005). The UNC-73/Trio RhoGEF-2 domain is required in separate isoforms for the regulation of pharynx pumping and normal neurotransmission in *C. elegans*. *Genes Dev* **19**, 2016-29.

Sze, J. Y., Victor, M., Loer, C., Shi, Y. and Ruvkun, G. (2000). Food and metabolic signalling defects in a *Caenorhabditis elegans* serotonin-synthesis mutant. *Nature* **403**, 560-4.

Tabara, H., Grishok, A. and Mello, C. C. (1998). RNAi in *C. elegans*: soaking in the genome sequence. *Science* **282**, 430-1.

Tavernarakis, N., Wang, S. L., Dorovkov, M., Ryazanov, A. and Driscoll, M. (2000). Heritable and inducible genetic interference by double-stranded RNA encoded by transgenes. *Nat Genet* **24**, 180-3.

Timmons, L. and Fire, A. (1998). Specific interference by ingested dsRNA. *Nature* **395**, 854.

Touroutine, D., Fox, R. M., Von Stetina, S. E., Burdina, A., Miller, D. M., 3rd and Richmond, J. E. (2005). *acr-16* encodes an essential subunit of the levamisole-

resistant nicotinic receptor at the *Caenorhabditis elegans* neuromuscular junction. *J Biol Chem* **280**, 27013-21.

Trent, C., Tsuing, N. and Horvitz, H. R. (1983). Egg-laying defective mutants of the nematode *Caenorhabditis elegans*. *Genetics* **104**, 619-47.

Tsalik, E. L., Niacaris, T., Wenick, A. S., Pau, K., Avery, L. and Hobert, O. (2003). LIM homeobox gene-dependent expression of biogenic amine receptors in restricted regions of the *C. elegans* nervous system. *Dev Biol* **263**, 81-102.

Waggoner, L. E., Dickinson, K. A., Poole, D. S., Tabuse, Y., Miwa, J. and Schafer, W. R. (2000a). Long-term nicotine adaptation in *Caenorhabditis elegans* involves PKC-dependent changes in nicotinic receptor abundance. *J Neurosci* **20**, 8802-11.

Waggoner, L. E., Hardaker, L. A., Golik, S. and Schafer, W. R. (2000b). Effect of a neuropeptide gene on behavioral states in *Caenorhabditis elegans* egg-laying. *Genetics* **154**, 1181-92.

Waggoner, L. E., Zhou, G. T., Schafer, R. W. and Schafer, W. R. (1998). Control of alternative behavioral states by serotonin in *Caenorhabditis elegans*. *Neuron* **21**, 203-14.

Weihe, E., Schutz, B., Hartschuh, W., Anlauf, M., Schafer, M. K. and Eiden, L. E. (2005). Coexpression of cholinergic and noradrenergic phenotypes in human and nonhuman autonomic nervous system. *J Comp Neurol* **492**, 370-9.

Weinshenker, D., Garriga, G. and Thomas, J. H. (1995). Genetic and pharmacological analysis of neurotransmitters controlling egg laying in *C. elegans*. *J Neurosci* **15**, 6975-85.

White J.G., S. E., Thomson J.N., Brenner S. (1986). The structure of the nervous system of the nematode *C. elegans*. *Philosophical Transactions of the Royal Society of London - Series B: Biological Sciences* **314**, 340.

White, J. G., Southgate, E., Thomson, J. N. and Brenner, S. (1976). The structure of the ventral nerve cord of *Caenorhabditis elegans*. *Philos Trans R Soc Lond B Biol Sci* **275**, 327-48.

Wolfe, J. M. and Van Wert, M. J. (2010). Varying target prevalence reveals two dissociable decision criteria in visual search. *Curr Biol* **20**, 121-4.

Wonnacott, S., Sidhpura, N. and Balfour, D. J. (2005). Nicotine: from molecular mechanisms to behaviour. *Curr Opin Pharmacol* **5**, 53-9.

Xiao, H., Hapiak, V. M., Smith, K. A., Lin, L., Hobson, R. J., Plenefisch, J. and Komuniecki, R. (2006). SER-1, a *Caenorhabditis elegans* 5-HT₂-like receptor, and a multi-PDZ domain containing protein (MPZ-1) interact in vulval muscle to facilitate serotonin-stimulated egg-laying. *Dev Biol* **298**, 379-91.

Yanik, M. F., Cinar, H., Cinar, H. N., Chisholm, A. D., Jin, Y. and Ben-Yakar, A. (2004). Neurosurgery: functional regeneration after laser axotomy. *Nature* **432**, 822.

Zengel, J. M. and Epstein, H. F. (1980). Identification of genetic elements associated with muscle structure in the nematode *Caenorhabditis elegans*. *Cell Motil* **1**, 73-97.

Zhang, M., Chung, S. H., Fang-Yen, C., Craig, C., Kerr, R. A., Suzuki, H., Samuel, A. D., Mazur, E. and Schafer, W. R. (2008). A self-regulating feed-forward circuit controlling *C. elegans* egg-laying behavior. *Curr Biol* **18**, 1445-55.

Zornik, E., Paisley, K. and Nichols, R. (1999). Neural transmitters and a peptide modulate *Drosophila* heart rate. *Peptides* **20**, 45-51.