Abstract

Effects of Nicotine on *Caenorhabditis elegans* survival, reproduction, and gene expressions–Development of an Invertebrate Animal Model for Drug of Abuse

by Michael A. Smith Jr

July, 2011

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DEPARTMENT OF BIOLOGY

Although much is known about the addictive effects of nicotine, the molecular mechanisms of nicotine-induced effects remain largely unclear. Specifically, little is known about the effects of nicotine on gene expression, including gene expression controlled by miRNAs. miRNAs may play a key role in regulating gene expression in response to nicotine exposure due to the fact that expression profiles of the miRNAs will be altered. These effects on gene expression may be associated with long-term use and the "addictive" behavior that is often exhibited with use of nicotine. Our goals are to explore the toxicity of nicotine on *Caenorhabditis elegans (C. elegans),* including survival, reproduction, and gene expression and to develop *C. elegans* as a model organism to assess the toxicity of various xenobiotics. We hypothesize that 1) Nicotine affects survival and reproduction *of C. elegans;* 2) The expression of several important genes, including the genes coding for the nicotinic acetylcholine receptor and for oxidative stress response, will be affected by nicotine exposure; and 3) The expression of miRNA genes will be changed and related to the selected protein coding gene expression. In survival trials, we tested a range of doses to obtain a 24- hour dose-response data. The 24-hour lethal dose-20 (LD20) in *C. elegans* corresponds to dose of ~3.16 ppm (19.5 uM) of nicotine. A reproduction study revealed that even at low nicotine exposure levels, egg-laying is affected.

Using qRT-PCR, we found that the expression of several egg-laying and oxidative stress related genes were altered by nicotine, which may be regulated by miRNAs. We were able to analytically determine that the expression patterns of the selected protein coding genes were dose-related. In the miRNA assay, we analyzed the expression of four miRNAs (Cel-mir-70, Cel-mir-58, Cel-mir-790, Cel-mir-253.), which were selected by *in-silico* prediction of miRNAs that potentially target our protein-coding genes of interest. We found that individual miRNA expression profiles varied among the different concentrations, indicating that the nicotine concentration induces a differential miRNA expression. At 3.16 ppm, where the protein-coding genes are the most active, miRNAs are also up-regulated indicating there is a complex system of regulation based on more than one miRNA, many miRNAs may target the same gene. Therefore, we believe that miRNAs may play a key role in controlling protein-coding gene expression. The understanding of this relationship between the toxicant (nicotine) and its effects on miRNAs and their targeted genes will lead to a greater understanding of mechanisms of nicotine-related addiction.

Effects of Nicotine on *Caenorhabditis elegans* survival, reproduction, and gene expressions–

Development of an Invertebrate Animal Model for Drugs of Abuse

A Thesis

Presented To

The Faculty of the Department of Biology

East Carolina University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science in Biology

by

Michael A. Smith Jr.

December, 2011

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ACKNOWLEDGEMENTS

I would like to thank Dr. Xiaoping Pan for giving me the opportunity to work in her lab. I have greatly appreciated her mentorship, encouragement, and support. I would also like to thank other members of my thesis committee Dr. Xiaoping Pan, Dr. Baohong Zhang, Dr. David Rudel, and Dr. Jamie DeWitt for their guidance and feedback.

Additionally, I would like to thank my colleagues in the Pan Lab, Zhang Lab, Dongliang Chen, and Yanqiong Zhang for their assistance and support. I would also like to thank the rest of the members of the Zhang lab, as well as the members of the Farwell and Pan Labs, for continued encouragement and support.

Finally, I would like to thank the Department of Biology at East Carolina University for financial support. This project was supported by the new-faculty startup fund of the Pan lab.

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LIST OF ABBREVIATIONS

C	Celsius
cDNA	complementary DNA
DNA	Deoxyribonucleic acid
dsRNA	Double stranded RNA
miRNA	microRNA
miRNA*	microRNA complementary sequence
mRNA	messenger RNA
NaCl	Sodium Chloride
nt	nucleotide
pre-miRNA	Precursor microRNA
pri-miRNA	Primary microRNA
pol II	RNA Polymerase II
qRT-PCR	Quantitative Real Time PCR
RNA	Ribonucleic acid
rRNA	ribosomal RNA
RISC	RNA Induced Silencing Complex
RT-PCR	Reverse transcription PCR
µg	Microgram

CHAPTER 1: INTRODUCTION

In 2008, nearly 71 million Americans aged 12 and older had used a tobacco product at least once in the month prior to being surveyed. The study also reported that 7-20% of 8th to 12th grade students had used a smokeless tobacco product in the month prior to being surveyed (Kaletta…). Nicotine is the primary substance in tobacco products that leads to addictive behavior. It is a major component of cigarette smoke, is contained in smokeless tobacco products, and is a large concern for the health care industry (Table 1-1) because it plays a key role in the development of lung cancer and cardiovascular disease (Yildiz 2004). Nicotine also is a central nervous system (CNS) stimulator (Picciotto…) that is involved in excitatory neurotransmission that affects many bodily functions.

Nicotine is an amine composed of pyridine and pyrrolidine rings (Figure 1-1). It has been shown that nicotine can cross biological membranes and the blood brain barrier easily (Oldendorf, Stoller et al. 1993). The absorbed nicotine is almost completely metabolized in the liver to form a wide selection of metabolites (Yildiz 2004). It has been shown to affect a large variety of functions including gene expression, regulation of hormone secretion, and enzyme activities (Yildiz 2004).

The primary target of nicotine is the nicotinic acetylcholine receptors (nAChRs) (Schafer 2002; Darsow, Booker et al. 2005). nAChRs are protein receptors that are located in the cellular membrane of the neuromuscular junction (Melstrom and Williams 2007). The nAChRs are included in a family of ligand-gated ion channels and contribute to many of the important processes in the brain, including cognitive and memory functions (Yu, Nordberg et al. 2003).

After being stimulated by nicotine, nAChRs regulate the function of pathways involved in many actions in the body. Some of these include: stress response, anxiety, and depression in the normal brain. Smoking and consequent uptake of nicotine can result in the changing of anxiety levels and mood (Yu, Nordberg et al. 2003). At the cellular level, stimulation of these nicotinic receptors leads to an increased synthesis and extracellular release of several hormones such as norepinephrine and epinephrine (Yildiz 2004).

Oxidative stress is one of the effects of nicotine exposure. Oxidative stress is defined as ‘‘a disturbance in the pro-oxidant-antioxidant balance in favor of the former, leading to potential damage’’ (Mary B. Newman and Thomas Tighea 2002). This imbalance can be caused by an increase in reactive oxygen species (ROS). Not much is known about the production of reactive oxygen species (ROS) upon nicotine exposure. The increase in ROS increases oxidative damage to the cellular membranes (Mary B. Newman and Thomas Tighea 2002). Mechanisms for coping with the oxidative stress include up regulation of radical detoxifying enzymes that are capable of converting free radicals to less toxic substances, such as glutathione - peroxidase, super-oxide dismutase (SOD), and catalase (Mary B. Newman and Thomas Tighea 2002).

Although much is known about the effects of nicotine on the nAChRs, little is known about its effects on specific nAChRs subunits, genes implicated in behavioral abnormalities, and potential microRNAs (miRNA) regulation of specific genes (Takahashi, Yamashita et al. 1999). miRNAs are short endogenous RNAs known to be major gene regulators. One study indicated that the miRNA regulated pathway is one of the cellular mechanisms involved in nicotine dependence (Li 2009). This study suggested that the DRD1 gene, which is shown to be involved in nicotine dependence, is differentially expressed when up regulated by miR-504 expression.

These effects on gene expression may be the cause of the "addictive" behavior that leads to long term use of nicotine.

Need for an animal model

Approximately 60%–80% of *C. elegans'* genes are conserved with humans (Vella 2005). This similarity suggests that *C. elegans* would be an appropriate model for understanding toxicological pathways at the gene or protein level in humans. It is important to note that while *C. elegans* and humans are similar in term of conserved genes, they are different in terms of pathological and phenotypical expression. There are several other characteristics that are shared between the phyla, including molecular and cellular pathways (Vella 2005). The entire genome sequence of *C. elegans* is known. Among the 18,452 known C. *elegans* protein sequences, at least 83% (15,344 sequences) have human homologous genes (Narahashi, Fenster et al. 2000).

In 1965 Sydney Brenner proposed *C. elegans* as a model organism for scientific research and it has been used for various types of studies, including: apoptosis, cell signaling, cell cycle, cell polarity, gene regulation and metabolism (Narahashi, Fenster et al. 2000). *C. elegans* are one of the most amenable, well-defined organisms that have a nervous system. This animal model is very easy to culture and maintain. The average adult worm is 1mm, they are free-living (non-parasitic), and can live on E.-coli lawned nematode growth medium (NGM). The small size of *C. elegans* allows in vivo assays to be performed. They have a short lifecycle/lifespan (3.5days/3 weeks), which allows for assays to be performed rapidly with larger numbers of organisms (Culetto and Sattelle 2000).

C. elegans are transparent, which allows the cell lineage and other parts of the body be easily studied under light microscopes. In addition, the known sequence of the entire genome

facilitates genetic manipulations for the creation of transgenic strains for particular studies (Culetto and Sattelle 2000).

Gonad development

In our studies, we plan to use larval stage 3 (L3) animals. For this reason it is important to understand how to identify *C. elegans* at various stages in development. One method that is used to determine the relative age of these worms is to look at the gonad development. This is called gonadogenesis, which starts approximately 7 hr after hatching. As seen in (Figure 1-2), the size and characteristics of the gonad vary at the different stages (Sulston and Brenner 1974; Hillier, Coulson et al. 2005). Figure 1-2 provides a cartoon representation of the development and characterization of the gonad cells. Primordial gonad cells are formed in L1. The four celled gonadal primordium (Z1-Z4) is shown in its midventral position in the newly hatched worm (Kimble and Hirsh 1979).

In hermaphrodite worms, the Z1 and Z4 cells give rise to the somatic structures of the gonad, and Z2 and Z3 give rise to the germ line cells (Kimble and Hirsh 1979). During the second half of L1, 12 cells are produced by the somatic gonad precursors Z1 and Z4 in the hermaphrodite worm (Kimble and Hirsh 1979). In hermaphrodites, the developing gonad elongates both anteriorly and posteriorly during L1, L2, and L3. The growing tips, as seen in (Figure 1-3), reflex around the time of the L3-L4 molt (Sulston and Brenner 1974; Hillier, Coulson et al. 2005). Germ cells increase in number in L2. The gonad has extended along the ventral body in L3. During L3-early L4, somatic gonad precursors yield a total of 143 cells, forming the anterior and posterior gonadal sheaths, the spermathecae and the uterus. The distal gonad arms continue their migration along the dorsal body wall muscles and by the L4/adult molt they complete their migration close to midline (Sulston and Brenner 1974; Hillier, Coulson

et al. 2005). At this time the vulva is open to the outside and uterus is full of fertilized eggs in the adult. At this stage, the hermaphrodite begins to lay eggs and completes its life cycle.

C. elegans microRNAs (miRNA)

The role of miRNAs in gene expression is to control a diverse range of processes, including cell fate specification, apoptosis, developmental timing, diseases, and metabolism (Sulston and Brenner 1974; Lee 2001). The very first miRNA identified in *C. elegans* was lin-4, discovered and reported by Victor Ambros and colleagues in 1993 (Vella 2005). Current literature indicates that 175 miRNAs have been identified in *C. elegans* so far (MIR-Base). Many of the *C. elegans* miRNAs are highly evolutionarily conserved across many species, i.e. from flies to humans (Lai, Chou et al. 2000; Narahashi, Fenster et al. 2000; Nass and Hamza 2007).

The transcription of the miRNA genes is performed by RNA polymerase II, resulting in primary miRNA transcripts (pri-miRNA) (Roush and Slack 2009). Similarly to the protein-coding targets of RNA Pol II, pri-miRNAs are processed into pre-miRNAs in the nucleus by the microprocessor complex (Denli, Tops et al. 2004) . The premiRNAs are then exported to the cytoplasm by Exportin 5 where they are further processed by Dicer into mature miRNAs (Yi, Qin et al. 2003; Dempsey, Mackenzie et al. 2005).

The mechanism for the biogenesis of the *C. elegans* miRNAs is a well-known process and occurs in a manner very similar to mammals. After the transcription of a miRNA gene, nuclear cleavage of the pri-miRNA by the Drosha RNase III endonucleases occurs. This enzyme cuts both strands of the pri-miRNA near the stem loop and generates ~60–70 nt stem-loop shaped miRNA precursor (pre-miRNA) (Sulston and Brenner 1974). This pre-miRNA is transported to the cytoplasm by the export receptor Exportin-5. The nuclear cut by Drosha

defines one end of the mature miRNA and cytoplasmic cut by Dicer, also RNase III endonuclease, defines the opposite one. Dicer recognizes the pre-miRNA and cuts both of its strands at about two helical turns away from the base of the stem loop. Then one of these ~ 22 nt miRNA duplex arms is chosen and mature miRNA is associated with RNA-induced silencing complex (RISC) (Vella 2005). Once incorporated into a cytoplasmic RISC, the miRNA will specify cleavage if the miRNA has sufficient complementarity to the mRNA, or it will repress productive translation if the miRNA does not have sufficient complementarity. These miRNAs down-regulate gene expression by interacting with partially complementary sequences in the 3' UTRs of their target genes (Sulston and Brenner 1974).

I hypothesize that nicotine exposure in C. elegans mediates miRNA gene expression dose-dependently, leading to cytotoxicity, decreased survival and reproduction, and alterations to protein coding gene expression.

To test this hypothesis, this project will identify dose dependent effects on survival and reproduction, and investigate differential expression patterns of both protein coding genes and miRNA genes after exposure to nicotine. My specific objectives are:

1. **Evaluate the cytotoxicity (represented by mortality) and reproductive effects (represented by egg-laying pattern) of nicotine exposure on *C. elegans* (Chapter 3)**

2. **Analyze selected protein coding gene expression in *C. elegans* after exposure to nicotine (Chapter 4)**

RT-PCR and TaqMan qRT-PCR assays were used to determine protein-coding gene expressions in *C. elegans* after exposure to nicotine.

3. Analyze selected miRNA expression in *C. elegans* after exposure to nicotine (Chapter 5)

RT-PCR and TaqMan qRT-PCR assays were used to determine miRNA gene expression profiles in *C. elegans* after exposure to nicotine.

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Gene (DRD1) Is Modulated by microRNA miR-504." BIOL PSYCHIATRY **65**: 702–705.

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Yu, W. F., A. Nordberg, et al. (2003). "Correlation of oxidative stress and the loss of the nicotinic receptor alpha 4 subunit in the temporal cortex of patients with Alzheimer's disease." Neurosci Lett 338(1): 13-6.

Table 1-1. The effects of nicotine in whole organisms and in cells

The effects in the whole organism	The effects at the cellular level
Increased heart rate	Increased synthesis and release of hormones
Cardiac contractility	Activation of thyrosine hydroxylase enzyme
Increased blood pressure	Activation of several transcription factors
Decreased skin temperature	Induction of heat shock proteins
Mobilization of blood sugar	Induction of oxidative stress
Increase in free fatty acids in the blood	Effects on apoptosis
Increased catecholamine levels in the blood	Induction of chromosome aberrations
Arousal or relaxation	Induction of sister chromatide exchange

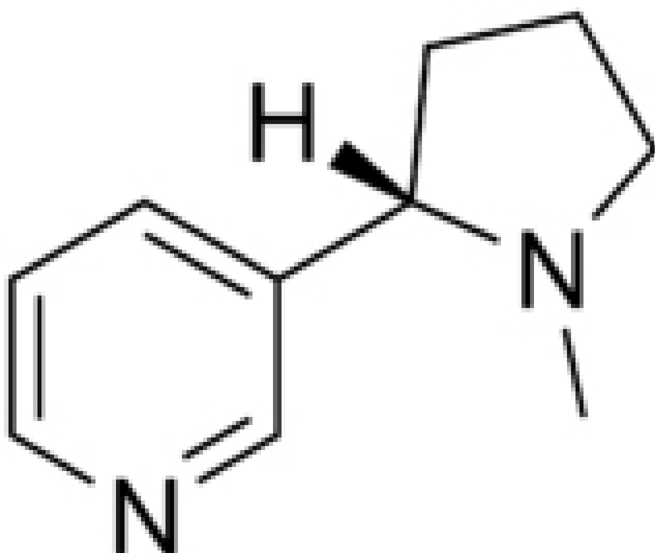
Yildiz, Deniz. "Nicotine, its metabolism and an overview of its biological effects." Toxicon 43 (2004): 619-32.

Table 1-2. Past Month Nicotine Dependence among Past Month Cigarette Users Aged 12 or Older, by Age Group, Race/Ethnicity, and Annual Family Income

	Past Month Nicotine Dependence among Past Month Cigarette Users	
Demographic Characteristic	**Percent**	**Standard Error**
Total	57.7	0.62
Age Group		
12 to 17	36.4	1.25
18 to 25	44.7	0.79
26 to 34	52.8	1.33
35 to 49	65.3	1.14
50 to 64	69.1	1.79
65 or Older	57.3	4.16
Race/Ethnicity*		
White	61.7	0.73
Black or African American	57.3	2.20
Asian	44.7	5.43
Two or More Races	57.2	5.02
Hispanic	38.2	2.06
Annual Family Income		
Less Than $20,000	63.0	1.21
$20,000 to $49,999	59.6	1.02
$50,000 to $74,999	54.6	1.78
$75,000 or More	49.6	1.52

Source: SAMHSA, 2006 NSDUH.

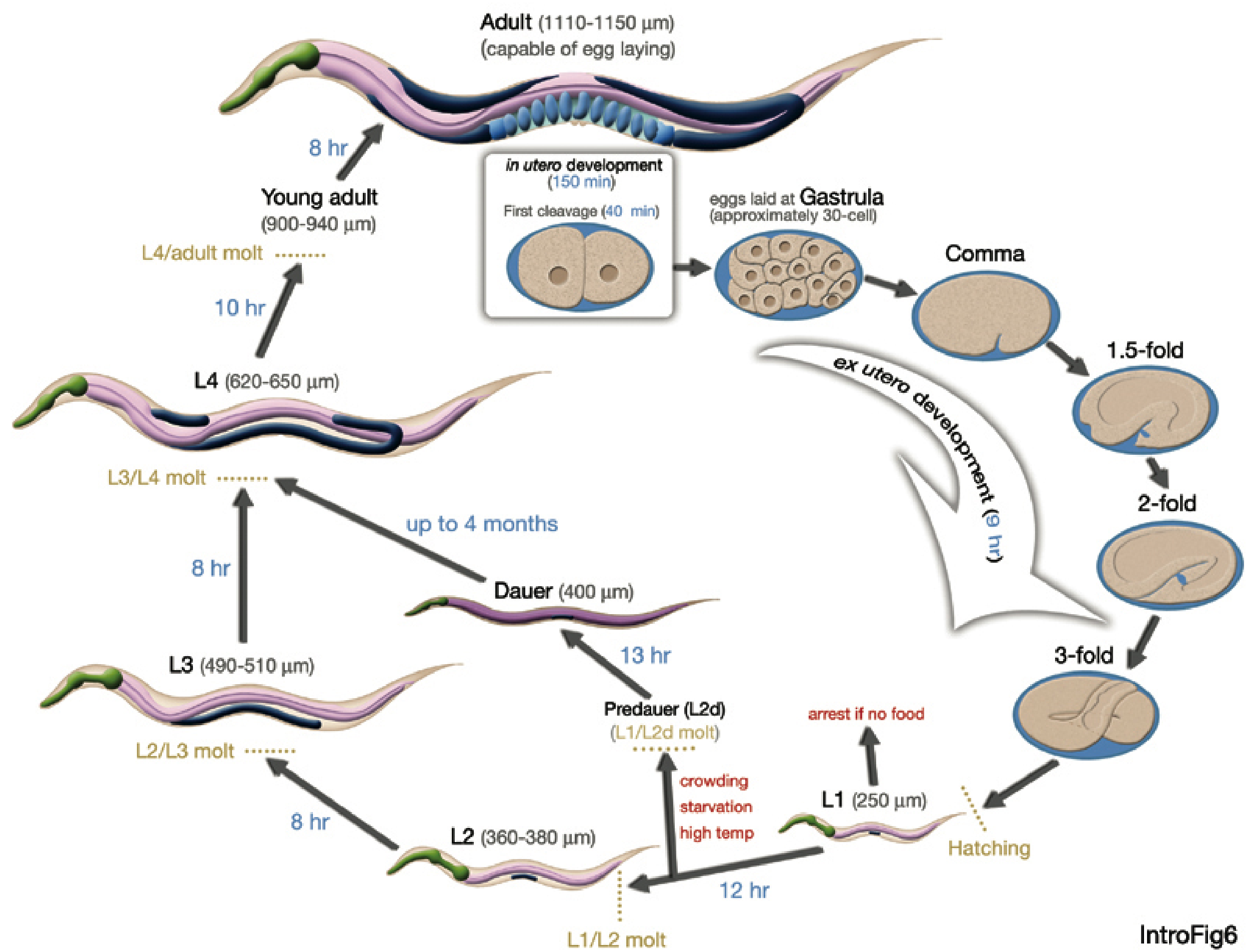
Substance Abuse and Mental Health Services Administration, Office of Applied Studies. (January 24, 2008). *The NSDUH Report: Nicotine Dependence: 2006*. Rockville, MD.



(Figure 1-1) Molecular structure of (+/-)-Nicotine ($C_{10}H_{14}N_2$)

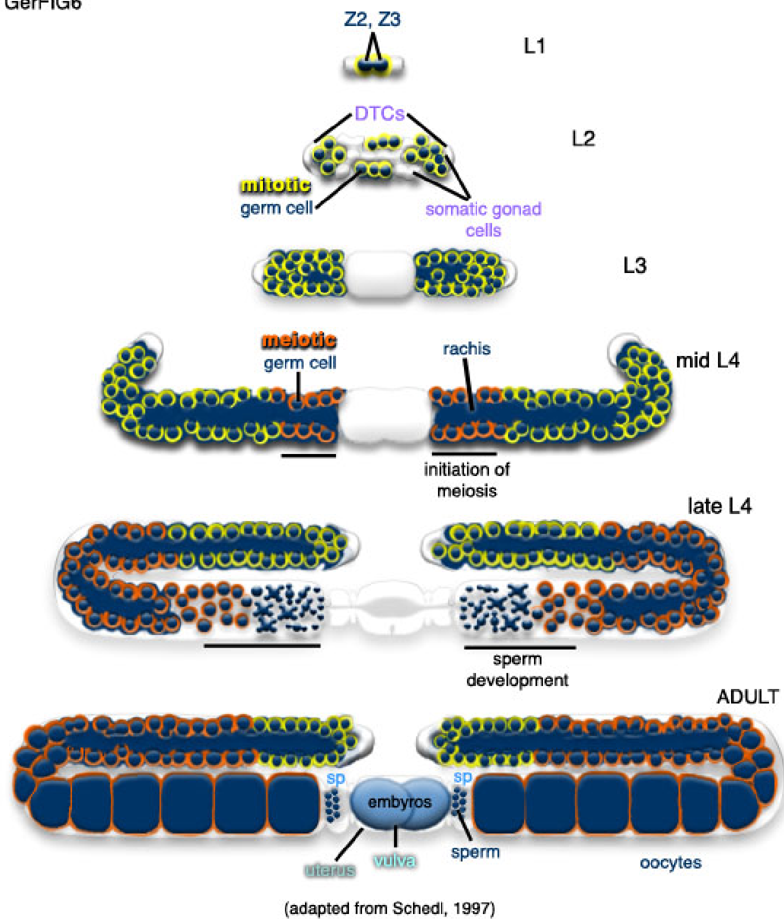
http://www.edinformatics.com/interactive_molecules/3D/nicotine_molecule.htm

[1]Hoffmann, Dietrich, and Ilse Hoffmann. "Chemistry and Toxicology." *Smoking and Tobacco Control Monograph No. 9*: 55-104. Web. 14 Mar. 2011. <http://dccps.nci.nih.gov/tcrb/monographs/9/m9_3.PDF>.



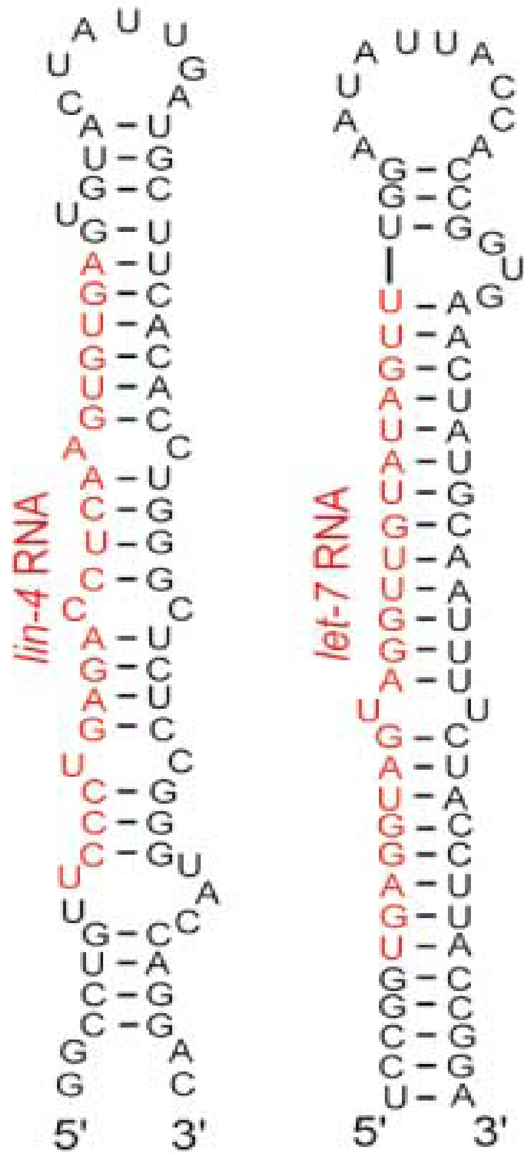
(Figure 1-2) C. elegans life cycle at 22^0C

CAENORHABDITIS Elegans AS A GENETIC ORGANISM." *Worm Atlas*. Web. 5 Oct. 2010. <http://www.wormatlas.org/ver1/handbook/anatomyintro/anatomyintro.htm>.

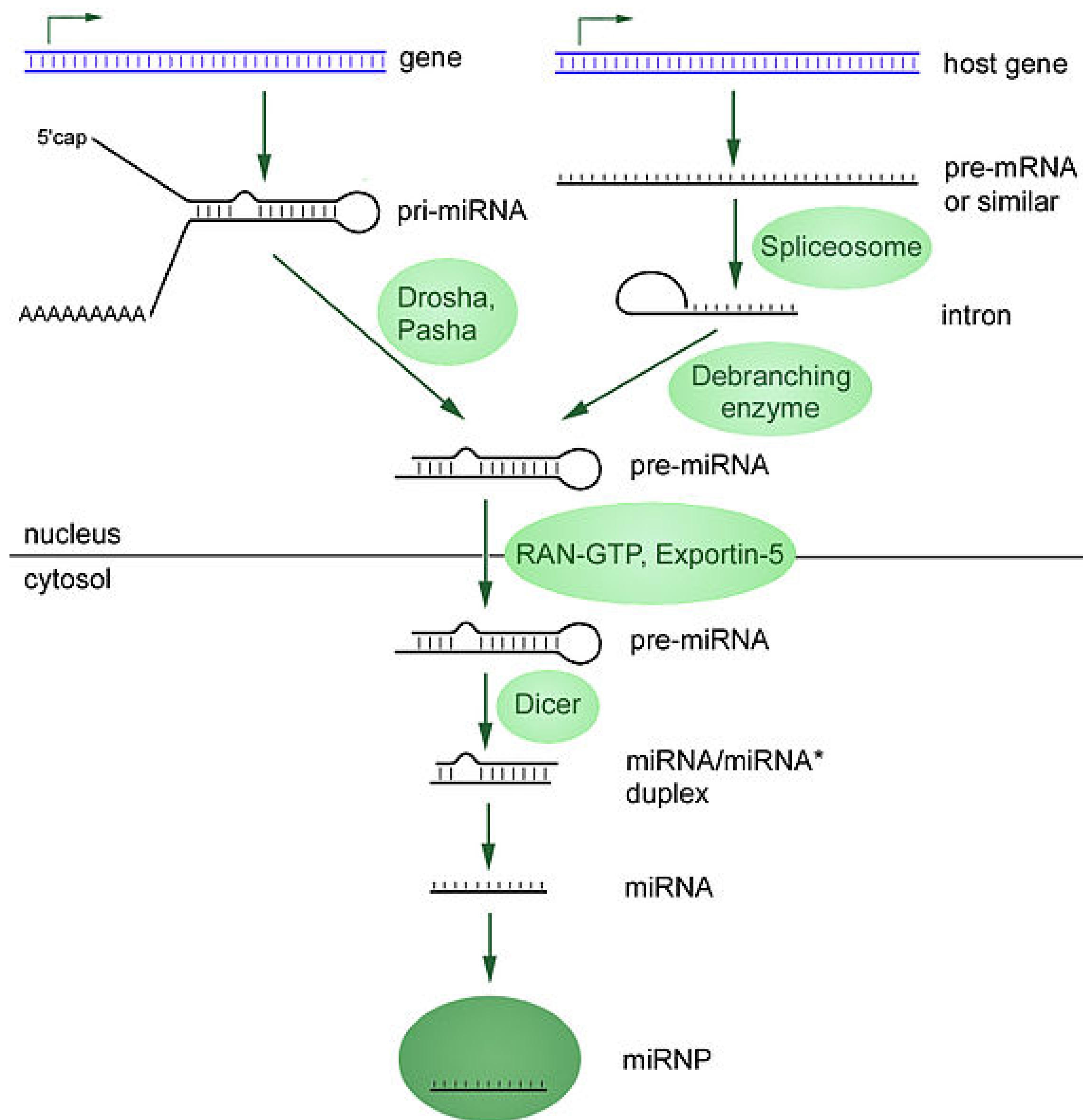


(Figure 1-3). Cartoon representation of post-embryonic hermaphrodite gonad development. Comparative size of gonads at different stages is not to scale.

WormBook: The Online Review of *C. elegans* Biology [Internet].



(Figure 1-4) Predicted stem loops of the founding miRNAs, *lin-4* and *let-7* RNAs (Lee et al., 1993;Reinhart et al., 2000). The precise sequences of the mature miRNAs were defined by cloning (Lau et al., 2001). Shown are the *C. elegans* stem loops, but close homologs of both have been found in flies and mammals (Pasquinelli et al., 2000; Lagos-Quintana et al., 2001, 2002).



(Figure 1-5). Biogenisis of miRNA

http://en.wikipedia.org/wiki/File:MiRNA-biogenesis.jpg

CHAPTER 2: Assay of 24 hour mortality in Caenorhabditis elegans following nicotine administration

Abstract:

Although much is known about the addictive effects of nicotine, the molecular mechanisms of the effects induced by exposure to nicotine remain ambiguous. The goal of this study was to explore the toxic effects of nicotine on *Caenorhabditis elegans (C. elegans)* and underlying molecular mechanisms that control many of the processes in the worm. This chapter reports the lethal effect of nicotine on *C. elegans*. A quantal dose response relationship, which is an all-or-none response, was performed to attain the 24 hour lethality of different nicotine doses. By modifying a previously established protocol (Williams 1999), we were able to synchronize and dose the L3 worms in order to obtain the lethal dose 20% (LD20) after exposure to various concentrations of nicotine for 24 hours (Stiernagle 2006). The results of this study show that there is a dose-response relationship of *C. elegans* mortality to nicotine exposure.

Introduction:

The quantal lethal dose assay is a standard toxicological assay that allows for the determination the lethal doses of toxicants in a given population. *Caenorhabditis elegans* is well suited for toxicological studies due to its established biology, short generation time, and large number of prodigy (Bischof, Huffman et al. 2006). Quantitative parameters of *C. elegans* that can be assayed include growth, size, progeny production, behavior, and mortality (Roh, Park et

al. 2009). The cellular complexity and the conservation of disease pathways between *C. elegans* and higher organisms, together with the simplicity and cost-effectiveness of cultivation, make for an effective *in vivo* model that lends itself to whole-organism assays. The next two chapters describe the reproduction and mortality assays we used to analyze the toxic effects of exposure to nicotine on *C. elegans.*

Materials and methods:

Synchronization

Wild type *C. elegans* strain Bristol N2 were grown in Petri dishes on 6-cm nematode growth medium (NGM) and fed with *Escherichia coli* strain OP50 according to a standard protocol (Brenner 1974). Worms at larval stage 3 (L3) from an age-synchronized culture were used in all experiments. To produce age-synchronized cultures, eggs from mature adults were isolated using a synchronization solution, followed by rinsing with M9 buffer (Roh, Park et al. 2009). After rinsing worms were placed on an NGM plate with no food for an arresting period to ensure all worm were closely synchronized. L3 worms were used because of their similarity in life cycle in relation to the teenage years in humans.

The worm were then transferred to OP50 seeded NGM agar plates, resulting in synchronized adult worm populations. L3 worms were washed out using M9, rinsed, and then centrifuged at 2000 rpm for 2 min at room temperature. A 2uL sample was taken and placed on a slide and counted under an inverse microscope to estimate the number of worms (Stiernagle

2006). Once we had the estimate of the number of worms per mL we calculated how many uL were needed to obtain ~50 worms per sample. The was performed in triplicate for each sample taken. This amount was then transferred to 1.5 mL microcenterfuge tubes with the dosing solution made of k-medium and the appropriate amount of nicotine. Each tube was labeled according to the dosage that was placed in that tube.

Preparation of Nicotine Treatment

A stock of 1,000 ppm nicotine was made from ACROSS organics L-nicotine, 98% pure (CAS 54-11-5) product. This was made by combining 10uL of L-nicotine with 9,990uL of K-media. Five doses were chosen to test: control (0ppm), 1ppm, 3.16ppm, 10ppm, 31.6ppm (which corresponds to 6.17µM, 19.5µM, 61.7µM, and 194.5µM, respectively)

C. elegans Treatment

Briefly, each test consisted of four concentrations and a control, in which ~50 L3 stage worms were transferred to 1.5mL microcenterfuge tubes with the dosing solution. Desired concentrations of doses were made in 1 mL quantities from the 1,000 ppm stock and the aliquots were placed in the corresponding 1.5 mL microcenterfuge tubes. These tubes were placed into a rotator in a 20^0C incubator at 42 rpm. The worms were exposed to the nicotine doses for 24 ± 0.5 hours.

Mortality Assay

After 24 hours the worms were collected, rinsed, and transferred to 12 well tissue culture plates filled with 1 mL NGM agar. The worms were allowed to acclimate to the new environment for one hour and then counted for survivability/mortality. The numbers of live and dead worms were determined via visual inspection using a dissecting microscope. If lethality could not be determined, the worms were probed with a platinum wire (Dhawan 1999). The worms that were not moving and did not respond to gentle probing were counted as dead. Statistical differences between the control and exposed worms were determined using standard statistical software (SPSS). Analysis of variance (ANOVA) was used for comparing means of different treatment groups. If there was a significant difference among treatment groups at $p < 0.05$ level, least significant difference (LSD) multiple comparisons were conducted to compare the mean of each group. The mean and standard deviation were also calculated.

Results:

Effects of Nicotine on C. elegans survivability

Figure 2-1 shows the results of the lethality vs. dose w. The results show that there is a dose response relationship between mortality and nicotine dosing. Increases in the doses of nicotine increased mortality by 4.37% (1ppm), 7.47 (3.16ppm), 14.53% (10ppm), and finally 20.61% (31.6ppm).

The linear model was performed on both the regular data and the logarithmic transformed dose data. The logarithmic dose transformation was preformed due to the fact out chosen dose correspond to a 0.5 log dose scale. The goodness of fit and significance of the regression model are shown in Table 2-1. All regression models for both the regular and the logarithmic

transformed dose data were concluded to be significant by p values of < 0.001. The correlation coefficient R^2 value for the regression models are as follows: regular dose data linear .831, log dose data linear 0.818.

Discussion:

Exposure to nicotine at a concentration greater than 3.16 ppm for 24 hour induced significantly higher mortality rate as compared to control ($P < 0.05$). The lethal effects followed a dose-response pattern as we defined four distinct statistical groups as nicotine concentration increased. The dose-response pattern is well described by a simple linear regression model, and LD 20 was determined at approximately 26 ppm (160.4 uM).

Studies that have shown that nicotine can activate nicotine-sensitive receptor conductance at concentrations as low as 1 mM (1000uM) (Richmond and Jorgensen 1999). The drug that is up taken through the *C. elegans* cuticle can vary as compared to that of its environment (Matta, Balfour et al. 2007). Therefore, it is critical to generate a dose–response curve on mortality especially at low concentrations where the genetic response may be evident.

The low dose effects of nicotine are important to investigate due to the fact that most smokers fall in to this range of nicotine exposure. The average full strength cigarette contains 1mg (equal to 55.5uM or 9ppm) of nicotine. The LD50 of nicotine is 40–60 mg (equal to 2220 uM or 360ppm) can be a lethal dosage for adult humans (Landoni 1991). Those statistics combined with the fact that the average number of cigarettes consumed by a smoker is 13-14 per day indicates an alarming amount of nicotine being administered to smokers' everyday if you multiply the amount of nicotine in one cigarette by the number smoked in one day the amount of

nicotine jumps to 721.5uM or 117 ppm. That is approximately one-third of the LD50 amount. These amounts of nicotine needed to observe these cytotoxic effects are higher in humans than the concentrations worked with in this study on *C. elegans* but understanding the cytotoxic effects at lower concentrations can give us a better idea of a safer range of exposure.

Conclusion:

In this study we performed the initial range-finding tests between 0 ppm and 31.6 ppm of nicotine. We concluded that there is a dose response relationship of *C. elegans* mortality and nicotine exposure. We determined the 24 hr LD20 of the toxicant nicotine as 26 ppm (160.4 uM).

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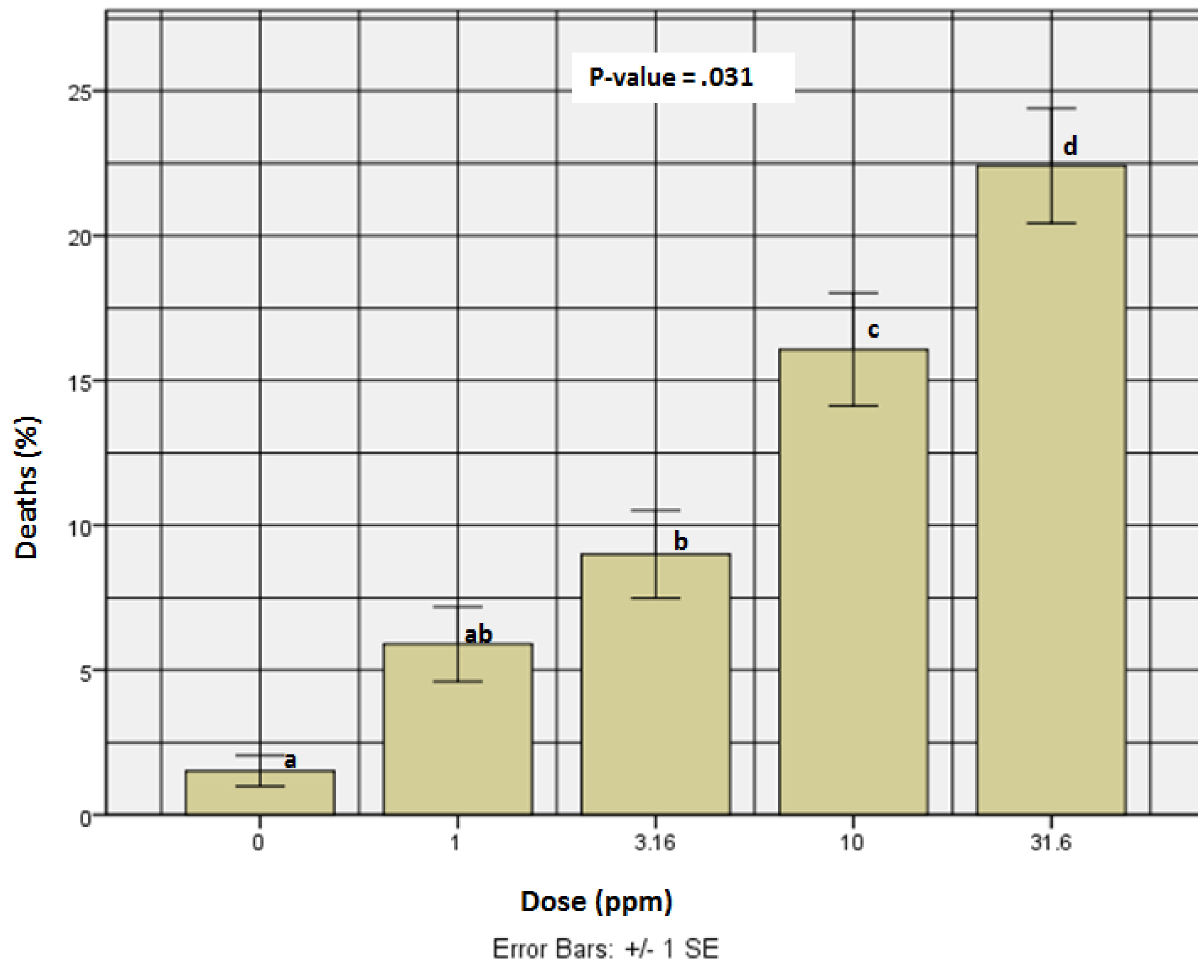

Figure 2-1. Lethality vs. Dose. Effects of different doses of nicotine on *C. elegans* mortality rate. L3 *C. elegans* were dosed for 24 hours then counted to obtain mortality. Around 50 worms were placed in each well in triplicate for one biological replicate. We preformed four biological replicates totaling ~600 worms for each dose. Error bars located on the bars indicate a 95% confidence interval. This means that 95% of the data is contained within the error bars, this works well to remove outliers that can affect the data. Statistical analysis was performed by the one-way Analysis of Variance (ANOVA) test using PASW Statistics 17 software. Different letters on bars indicated distinct statistical groups. The mortality of > 3.16 ppm groups is significantly higher than the control.

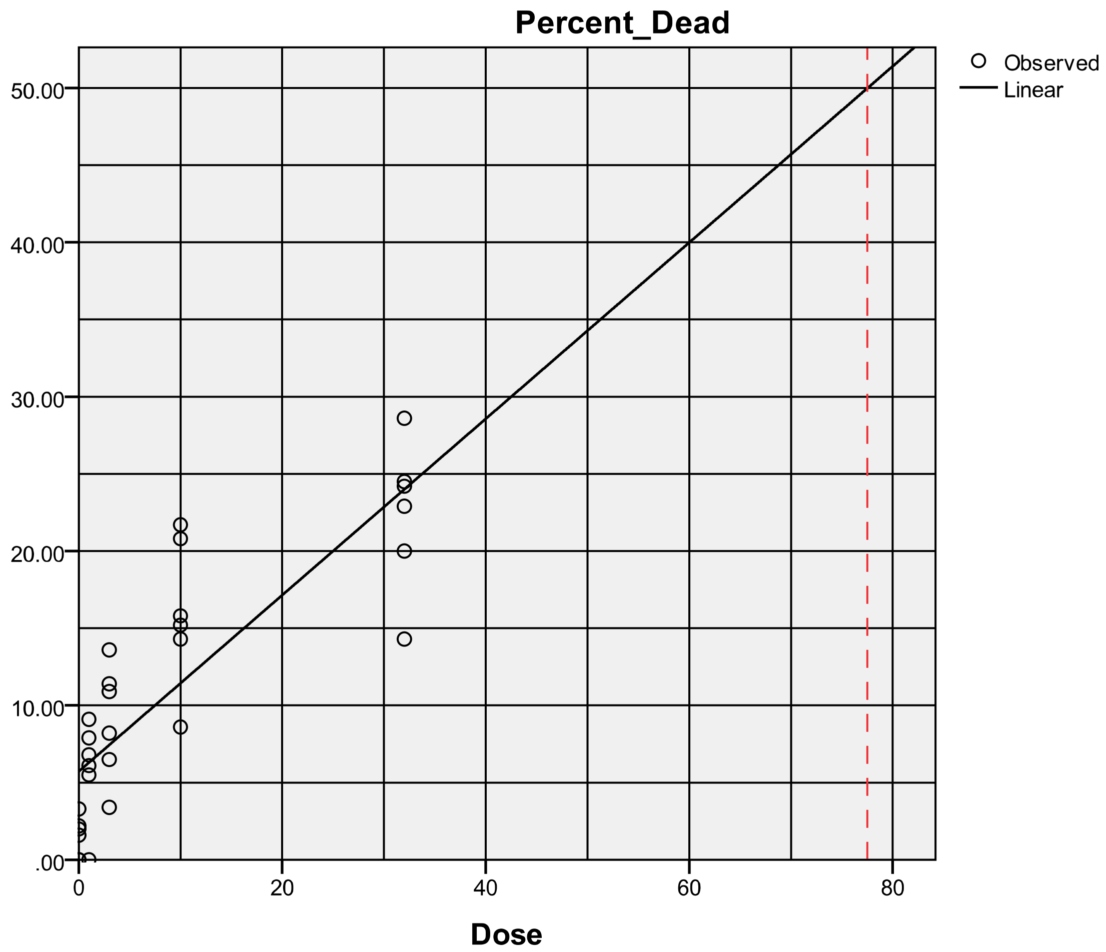

Figure 2-2. Lethality vs. Dose. Both Linear and Quadratic regression models of the lethality of nicotine in *C. elegans.* Correlation and significance can be found in Table 2-1

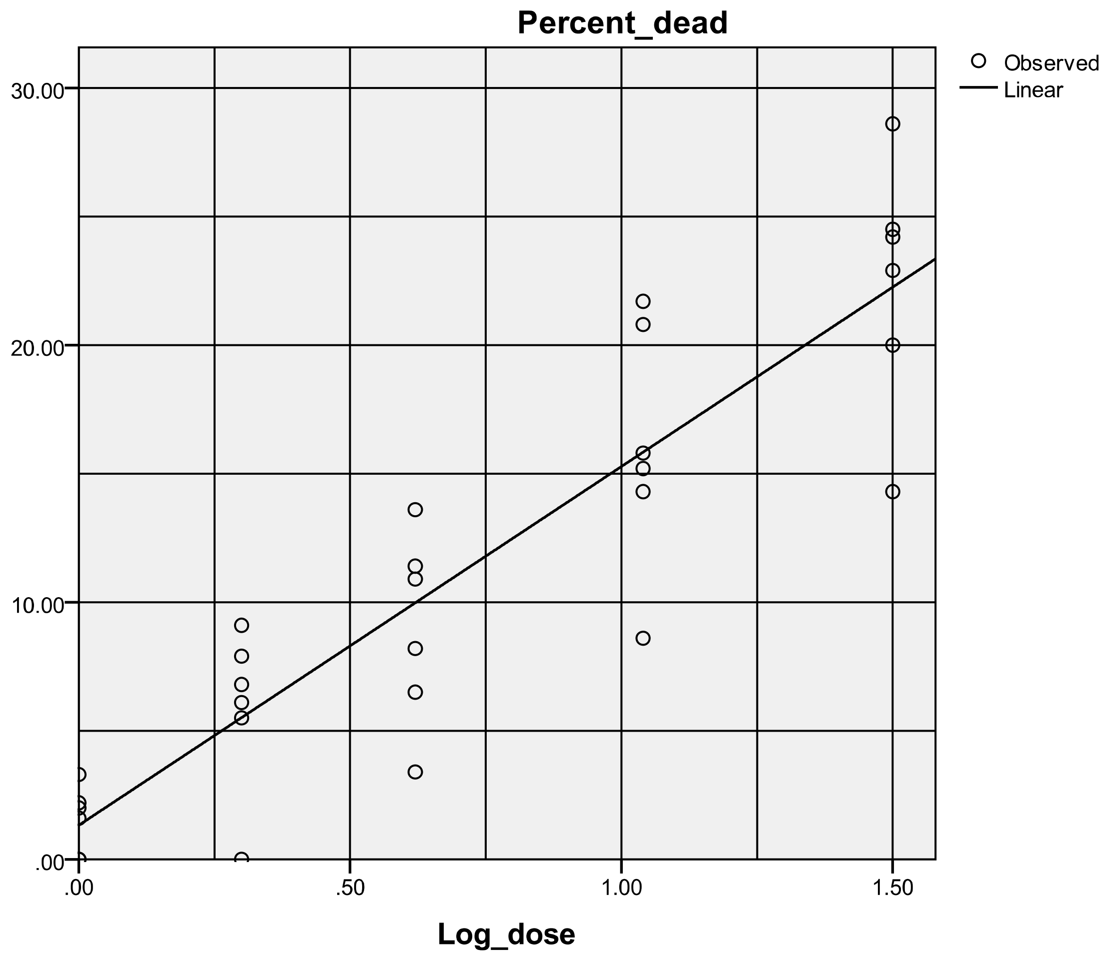

Figure 2-3. Lethality vs. Log Dose. Both Linear and Quadratic regression models of the lethality of nicotine in *C. elegans.* Equations, correlation, and significance can be found in Table 2-1

Table 2-1 Linear and Quadratic Regressions for the regular and logarithmic data sets

	Regression method	Equation	R^2	Sig.
Regular dose data	Linear	y=.57x + 5.7	.831	.000
Logarithmic dose data	Linear	y=13.95x + 1.32	.818	.000

CHAPTER 3: *C. elegans* egg laying and viability assay

Abstract:

Although much is known about the addictive effects of nicotine, the molecular mechanisms of nicotine-induced effects remain largely unclear. Our goals are to explore the toxicity effects of nicotine on *Caenorhabditis elegans (C. elegans).* In this chapter of the study we look at the effects of the nicotine dosing on egg-laying and viability. One of the primary targets of nicotine is nicotinic acetylcholine receptors (nAChRs) which mediate fast neurotransmission in nerves and muscles, some of which control the sex-specific muscles involved in *C. elegans* egg lying(Kim, Poole et al. 2001; Mongan, Jones et al. 2002). After exposing the *C. elegans* to various concentrations of nicotine, we performed an egg laying assay, counting the number of eggs laid by each worm. Each worm was placed in a separate well of a 24-well tissue culture plate. Eggs were counted every 12 hours for the duration of the egg laying event.

Introduction:

One of the most important behaviors for the analysis of nicotine toxicity in *C. elegans* is the mechanism of egg-laying. *C. elegans* hermaphrodites are self-fertile; they produce both sperm, which is stored in the spematheca, and oocytes (Schafer 2005). Egg-laying in *C. elegans* occurs within the first day of the L4/adult molt, the fertilized eggs are stored in the uterus, and a young adult hermaphrodite will generally have a store of 10–15 eggs in its uterus at any given time (Schafer 2005). Once egg-laying occurs, eggs are expelled from the uterus through the

contraction of 16 vulval and uterine muscles (White 1986; Kim, Poole et al. 2001). When in the presence of favorable conditions (food, temperature, and space), wild-type animals lay eggs in a specific temporal pattern: egg-laying tends to be clustered in short bursts, or active phases, which are separated by longer inactive phases during which eggs are not laid (Wei Geng 2005). Many neurotransmitters and neuronal signal transduction pathways have been shown to have specific effects on egg laying behavior; therefore it has become an important behavioral assay for the analysis of many toxicological effects on such mechanisms in *C. elegans* (Wei Geng 2005).

Egg-laying occurs through a simple movement involving specialized smooth muscle cells, when the muscles contract, they open the vulva and compress the uterus so that eggs can be laid in the environment. The egg-laying muscles are controlled by two classes of motor neurons (White 1986; Chen, Harris et al. 2005). The egg laying event occurs as the worms become young adults ~65 hr after hatching at 20^{0}C (Altun 2009). The event lasts for ~63 hr (2.5 days) and eggs are laid in clustered episodes with gaps between the events (Wei Geng 2005). Egg-laying behavior in *C. elegans* is regulated by multiple neurotransmitters, including acetylcholine and serotonin (Dempsey, Mackenzie et al. 2005).

Nicotine is a known target substrate for the nicotinic acetylcholine receptors (nAChRs) in the mammalian CNS and subunits of these receptors have also been identified in *C. elegans*. Nicotinic acetylcholine receptors (nAChRs) mediate fast excitatory neurotransmission in neurons and muscles. There are a total of 13 nAChR α subunits that control many functions such as signal transmission, locomotion, and muscle contraction (Gottschalk, Almedom et al. 2005).

Materials and methods:

Synchronization

C. elegans were grown in Petri dishes on 6 cm nematode growth medium (NGM) and fed OP50 strain *Escherichia coli* according to a standard protocol (Brenner 1974). Laval stage 3hermaphrodite animals from an age-synchronized culture were used in all the experiments. To produce age-synchronized cultures, at 2–3 days, eggs from mature adults were isolated using a synchronization solution, followed by rinsing with M9 buffer (Hitchcock 1998). After rinsing worms were placed on an NGM plate with no food for an arresting period to ensure all worm were closely synchronized.

The worms were then transferred to NGM agar plates with a food source, resulting in synchronized adult worm populations. L3 larval stage worms were washed out using M9, rinsed, and then centrifuged at 2000 rpm for 2 min at room temperature a 2uL sample was taken and placed on a slide and counted under an inverse microscope to estimate the number of worms.

Preparation of Nicotine Treatment

A stock of 1,000 ppm nicotine was made from ACROSS organics L-nicotine, 98% pure (CAS 54-11-5) product. This was made by combining 10uL of L-nicotine with 9,990uL of distilled water. Five doses were chosen to test: control (0ppm), 1ppm 3.16ppm, 10ppm, 31.6ppm.

C. elegans Dose-Response Treatment

Briefly, each test consisted of four concentrations and a control, in which ~50 L3 stage worms were transferred to 1.5 mL microcenterfuge tubes with the toxicant in each tube. Each was made in 1 mL quantities from the 1,000ppm stock and placed in the corresponding 1.5 microcenterfuge tubes with the ~50 worms. These tubes were placed into a rotator at 40 rmp in an incubator at 20^{0}C. The worms were exposed to the nicotine doses for 24 hours.

"Picking" method of transfer

After 24 hours the worms were collected, rinsed, and transferred to fresh NGM plates so that they could be picked and placed in the wells of tissue culture plates. The picking method was used to pick one animal at a time. The worm picker was made by mounting a 1-inch piece of 32 gauge platinum wire into the tip of a Pasture pipet. The end of the wire was flattened slightly with a hammer. To pick the worm, the tip of the wire was coated with a blob of E. coli OP50 and then touched gently to the top of the chosen worm, which stuck to the bacteria. The picked worm was transferred to the corresponding well on a 24 well NGM filled tissue culture plate, the tip was slowly lowered and gently touched the surface of the agar, and the worm was allowed to crawl off of the picker (Stiernagle 1999).

Method of C. elegans egg-laying and viability assay analysis

Once the worms had been "picked" and transferred to their respective wells, eggs that were laid and eventual hatched larvae were counted every 24 hrs for the duration of the egg laying period. The egg laying event lasts for ~63 hr (2.5 days) and eggs are laid in clustered episodes with gaps between the events (Wei Geng 2005).

Results:

The results of this study are shown in Figure 3-1 and Figure 3-2. Dosed L3 worms were placed into individual wells on a 24-well cell tissue culture plate (one worm per well) that was seeded with E-Coli. OP-50 as food. Eggs and larvae were counted at 24, 48 and 72 hrs after dosing to obtain the number of eggs laid by each worm. Statistical analysis was performed by the one-way Analysis of Variance (ANOVA) test using PASW Statistics 17 software.

Figure 3-1 represents the number of eggs and larvae counted at each time point in each well minus the previously counted number, i.e. the values of time point 48 hour represent the number of newly laid eggs during the period of 24-48 hours post-dosing. . Statistical significance among different dosed groups was tested using SPSS within each time point. During 0 -24 hours post-dosing, none of the dosed groups showed a significant difference from the control ($p > 0.05$). During 24-48 hours post-dosing, the 10ppm dosed group showed a significantly higher number of eggs plus hatched larvae as compared to control ($p = 0.015$). No significant difference was found in other dosed groups. No other dosed groups were different from control

during 24-48 hours post-dosing. For the period of 48-72 hours post-dosing, there was no significant difference in eggs-laid of dosed groups as compared to control.

Figure 3-2 represents the total number of eggs and larvae counted at each time point in each well. Statistical significance was measured between different dosed groups within each time point. At time point 24hr only one of the doses showed a statistically significant difference from the control. Eggs laid by the 10 ppm group were significantly higher than control ($p= 0.049$). The remaining dosed groups were not significantly different as compared to control group.

The accumulated eggs-laid from 0-48 hours post-dosing were counted at time point 48hr. The result is similar to that of the 24hr count. Again the 10 ppm dosed group showed significantly higher eggs-laid ($p= .034$) as compared to control group. No differences were found in the remaining doses.

The accumulated eggs laid from 0-72 hours post-dosing were counted at time point 72hr. The accumulated egg-laid pattern was slightly different from the previous time points. The accumulated egg-laid by worms in the highest concentration group (31.6 ppm) were significantly higher than the control ($p= 0.048$), whereas other dosed groups had no significant differences.

Figure 3-3 gives a graphical representation of total eggs laid vs. time. Three regressions were made on this graph: linear, quadratic and logarithmic. For each of the regressions an equation, R^2, and a significance value were calculated; results of these calculations can be found in table 3-1. The three regressions gave similar results, each had a great correlation due to the R^2 values: 0.970, 0.977, and 0.973 respectively. Each also had a significance of < 0.001. Due to this similarity, the simplest model linear regression is sufficient to describe the egg-laying pattern during 0-72 hours post-dosing.

Discussion:

The egg laying patterns of C. *elegans* post nicotine exposure were studied over the period of 0-72 hours post-dosing. The pattern of egg lying was observed and charted in Figure 3-1 show the eggs laid pattern as divided into three time periods (0-24 hour, 24-48 hour, and 48-82 hour). Figure 3-2 shows the accumulated eggs laid over time (0-24 hour, 0-48 hour, and 0-72 hour). The overall temporal pattern of egg-laid in *C. elegans* is clustered and fitted into three regression models and is shown in Figure 3-3. There are short periods of high-frequency egg-laying behavior; these short periods are called active states. During these active states approximately 1 egg is laid a minute for 2-10 min. These egg laying events are separated by much longer periods of approximately 20 minutes to 1 hour long where no eggs are laid; these events are called inactive states (Zhang, Schafer et al.). From our data, the average number of eggs laid after 72 hr, approximately the completion time of the egg laying events, was 268.29 this translates to 3.77 eggs/hr. This falls within the range of the acceptable egg laying activities of the natural egg lying patterns of the worms (Zhang, Schafer et al.).

Our results indicate that nicotine exposure for 24 hours changed the egg-laying pattern at some doses. More eggs were laid during the 24-48 hour period at 10ppm group (Figure 3-1), although the total eggs laid by this group was not significantly different from control during the whole 0-72 hour period (Figure 3-2). The significantly higher egg-lying of the 10 ppm group during 0-48 hours may be attributed to the higher rate of egg-lying in the 24-48 hour section. More eggs were laid by the 31.6ppm dosed group as compared to control during the whole 72

hour period (Figure 3-2). This finding is consistent with the literature that nicotine stimulates egg-laying and likely does this by activating the nicotinic acetylcholine receptors.

From our data we generally can conclude that the effects of nicotine on egg laying are not measurably different than the control, until we reach the higher end of our dosing range (10 ppm and 31.6 ppm). Results from other studies of the number of eggs laid after exposure to 64.8 ppm (400 µM) yield 3 worms/2 hours resulting in only ~98 worms over the course of the egg-laying period (Matta, Balfour et al. 2007). The decrease in the number of eggs being laid could possibly be due to the fact that nicotine is stimulating the egg-laying muscles in hypercontractivity and therefore decreaseing the number of eggs laid

Regressions were performed on the data to study the relationship between time and the number of eggs laid. Each of the regressions, (Figure 3-3 and Table 3-1), give a strong positive correlation (R^2 close to 1), so we can conclude that as time increases the number of eggs also increases. This was expected, but does not fully describe our results. Figure 3-1 shows the number of eggs laid minus the pervious time point's count. As seen in Figure 3-1, there is an increase in the number of eggs laid from time point 24hr to 48hr and then a slight decrease at time point 72 hr. This indicates that the majority of the egg laying is occurring between time points 24hr and 48hr. This study does not give a direct correspondence between human pathology and *C. elegans* phenotypes. Many mammalian models are often not reliably predictive of drug action in humans; it is merely a preclinical model perspective. Humans do not exhibit the phenotype of egg-laying as *C. elegans* do, but some of the genes that are associated with the egg-laying behavior are known to mediate signaling pathways in humans. One example would be *acr-16. It* has been reported to function as a nAChR in the neuromuscular junction (Touroutine, Fox et al. 2005). It should be noted that nicotine can affect muscle function under certain

conditions. For example, high concentrations of nicotine paralyze worms by acting in the muscle (Gottschalk, Almedom et al. 2005). While under our conditions, nAChRs seem to primarily act in neurons to mediate their function in nicotine responses.

Conclusion:

The conclusion of this study is that nicotine is a known effecter of some of the nicotinic receptors that deal with egg laying patterns, and there seems to be a slight increase in the number of eggs laid after exposure to higher concentrations of nicotine (10 ppm, 31.6 ppm) Also, even though there are statically significant values indicating that there is an effect on the egg-laying by time, additional time points are needed to accurately evaluate the validity of this conclusion. Studies with higher dosing ranges could also give a much clearer view to some of these possible effects.

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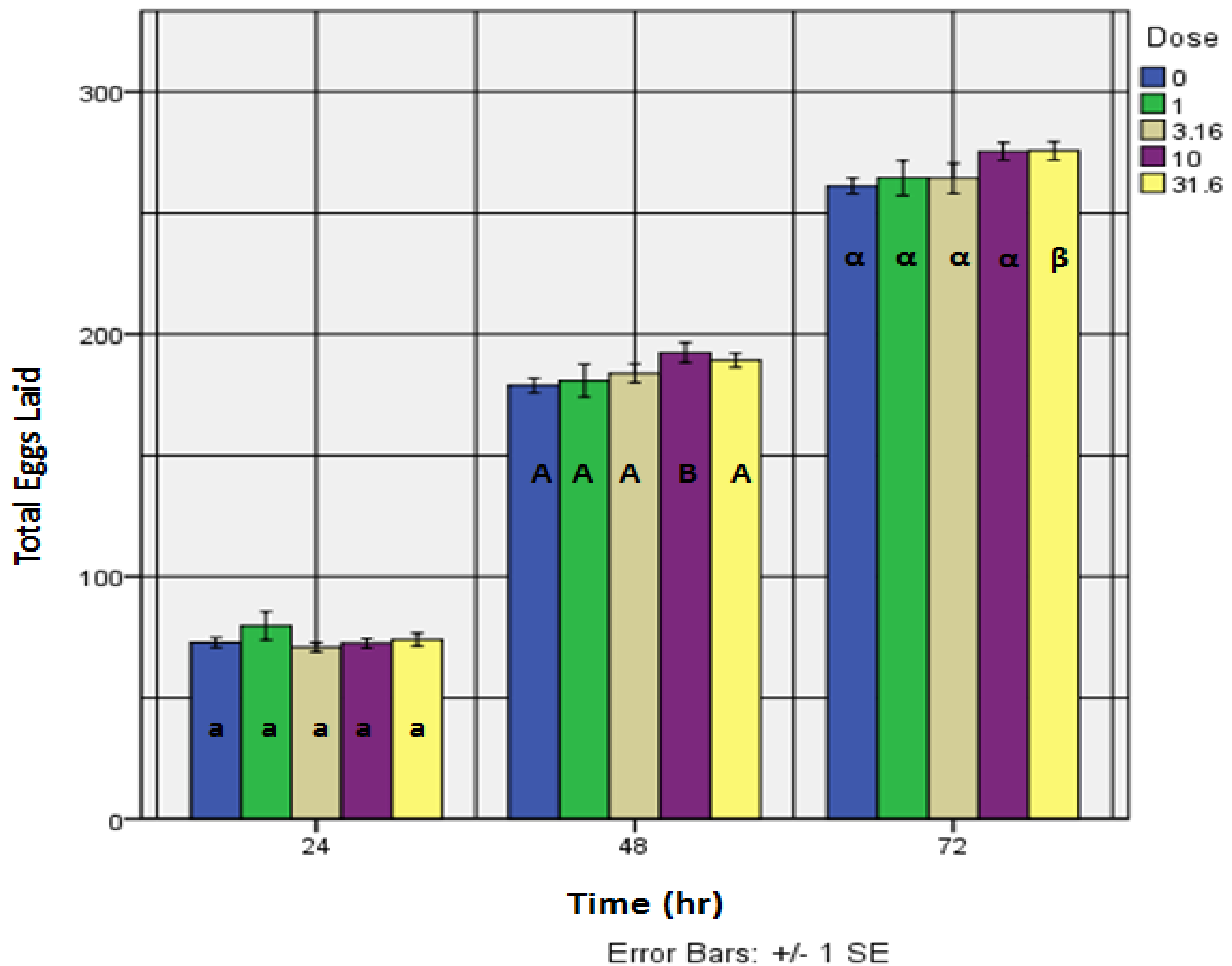


Figure 3-1. Eggs Laid vs. Time. Effects of different doses of nicotine on *C. elegans* egg-laying rate. *C. elegans* were dosed for 24 hours then placed in individual wells to lay eggs. Eggs were counted at time points 24, 48, and 72 hr after being dosed. Data at the time point 24hr represent the number of egg laid during the period of 0-24 hr. Data at the time point 48hr represent the number of egg laid during the period of 24-48 hr. Data at the time point 72hr represent the number of egg laid during the period of 48-72 hr. This significance is indicated on Figure 3-1 as an uppercase "B" as compared to an uppercase "A" to show the difference from the control.

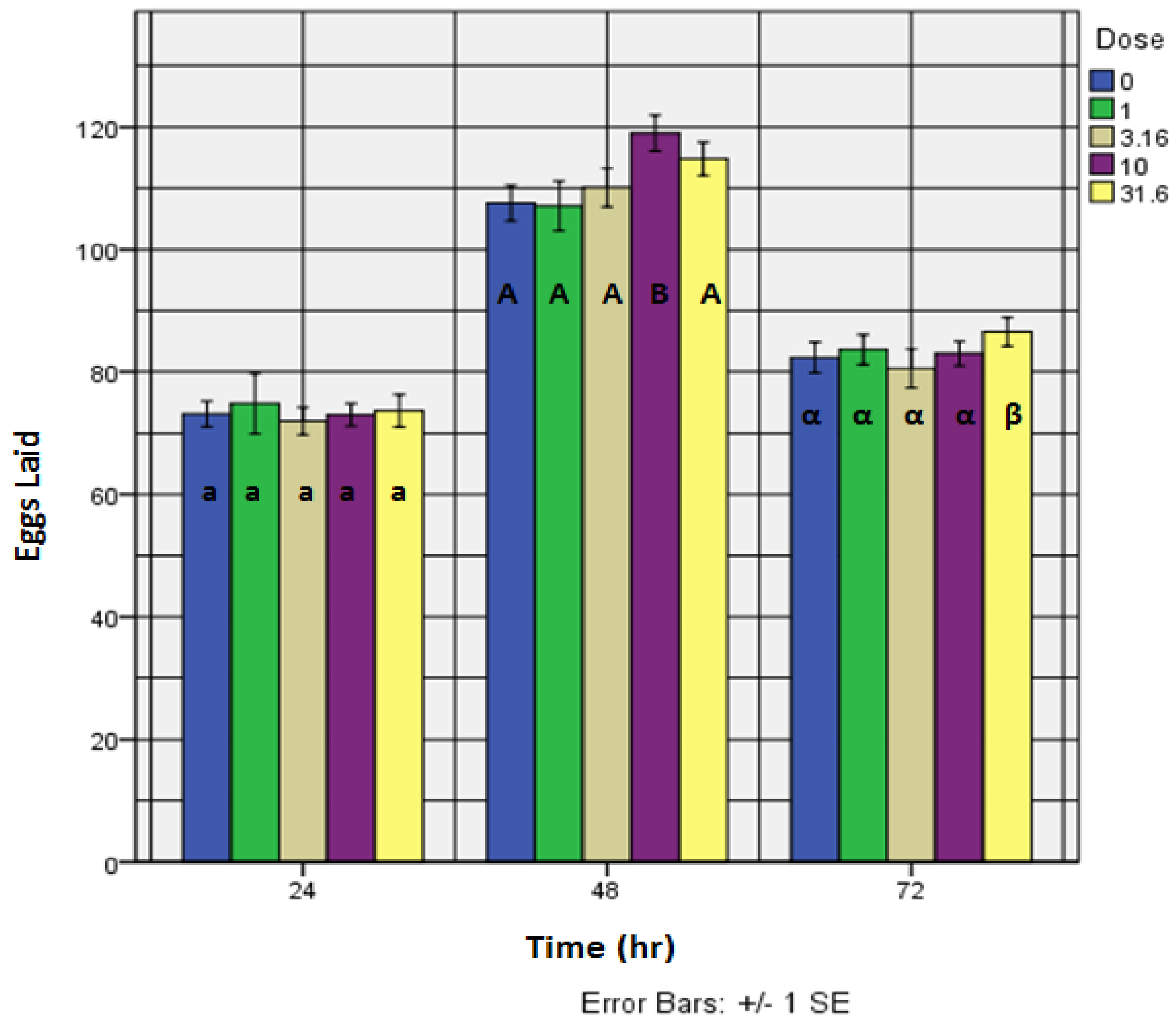
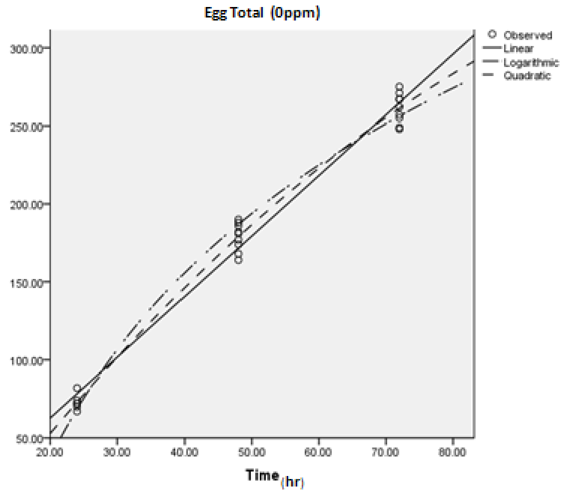
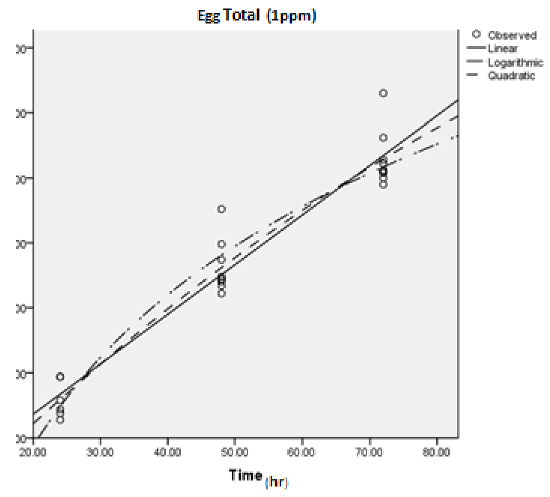
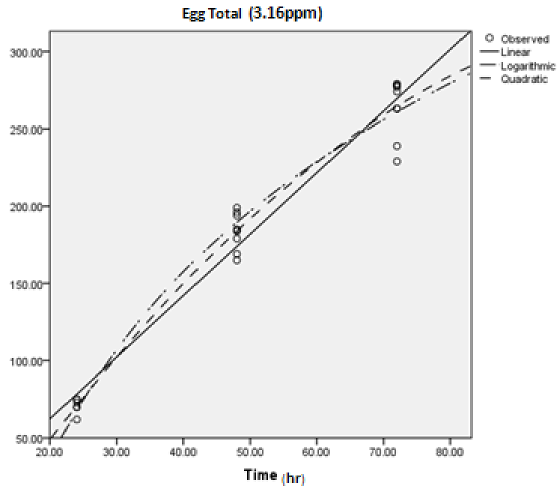
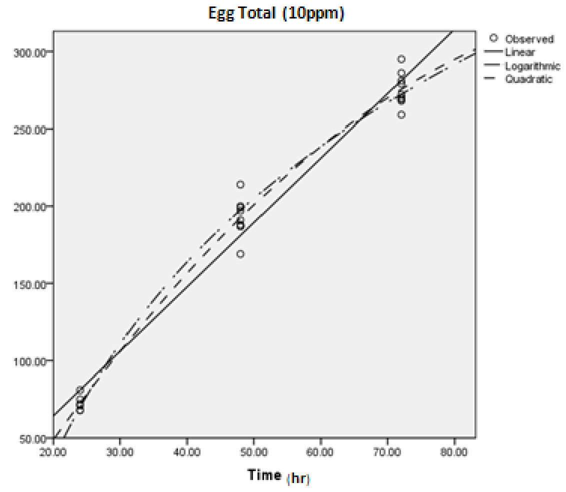


Figure 3-2. Total Eggs Laid vs. Time. Effects of different doses of nicotine on *C. elegans* total egg laying rate. *C. elegans* were dosed for 24 hours then placed individually in 24-well plate to allow egg-laying. Eggs were counted at time points 24, 48, and 72 hr after being dosed.Data at the time point 24hr represent the number of egg laid during the period of 0-24 hr. Data at the time point 48hr represent the number of egg laid during the period of 0-48 hr. Data at the time point 72hr represent the number of egg laid during the period of 0-72 hr.


Egg Total (0ppm)
Observed
Linear
Logarithmic
Quadratic
300.00
250.00
200.00
150.00
100.00
50.00
20.00
30.00
40.00
50.00
60.00
70.00
80.00
Time (hr)


Egg Total (1ppm)
Observed
Linear
Logarithmic
Quadratic
20.00
30.00
40.00
50.00
60.00
70.00
80.00
Time (hr)


Egg Total (3.16ppm)
Observed
Linear
Logarithmic
Quadratic
300.00
250.00
200.00
150.00
100.00
50.00
20.00
30.00
40.00
50.00
60.00
70.00
80.00
Time (hr)


Egg Total (10ppm)
Observed
Linear
Logarithmic
Quadratic
300.00
250.00
200.00
150.00
100.00
50.00
20.00
30.00
40.00
50.00
60.00
70.00
80.00
Time (hr)

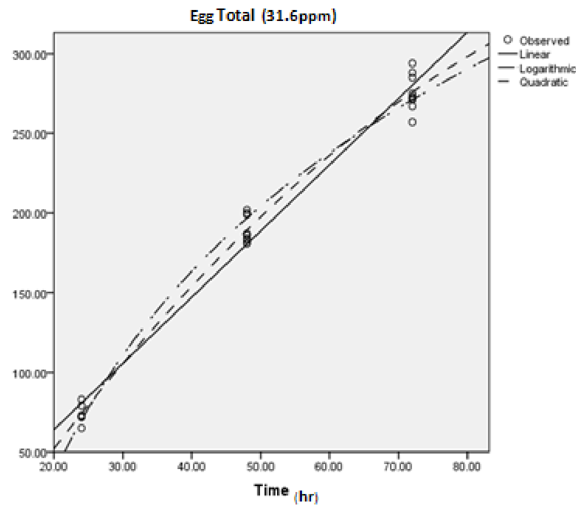

Egg Total (31.6ppm)
Observed
Linear
Logarithmic
Quadratic
300.00
250.00
200.00
150.00
100.00
50.00
20.00
30.00
40.00
50.00
60.00
70.00
80.00
Time (hr)

Figure 3-3. Total Eggs Laid vs. Time. The total number of eggs that were laid at each time point and each dose were plotted to get a regression in order to correlate the effects of time and the different doses of nicotine on *C. elegans* total egg laying rate. *C. elegans* were dosed for 24 hours then placed in individual wells to lay eggs. Eggs were counted at time points 24, 48, and 72 hr after being dosed.

Table 3-1 The Linear, Quadratic, and logarithmic Regressions for each dose of the total egg laying data set

Dose	Regression method	Equation	R^2	Sig.
Total egg laying data (0ppm)	Linear	3.88 * x + -14.97	.982	<.001
	Logarithmic	-474.77 + 170.91 * log(x)	.981	<.001
Total egg laying data (1ppm)	Linear	3.82 * x + -8.05	.936	<.001
	Logarithmic	-485.35 + 167.61 * log(x)	.929	<.001
Total egg laying data (3.16ppm)	Linear	3.98 * x + -17.22	.961	<.001
	Logarithmic	-491.11 + 175.83 * log(x)	.968	<.001
Total egg laying data (10ppm)	Linear	4.17 * x + -19.03	.972	<.001
	Logarithmic	-516.24 + 184.37* log(x)	.982	<.001
Total egg laying data (31.6ppm)	Linear	4.15 -19.17	.980	<.001
	Logarithmic	-512.19 + 183.14 * log(x)	.982	<.001

CHAPTER 4: protein-coding gene expression analysis

Abstract:

Having a completely sequenced genome, such as that of *Caenorhabditis elegans (C. elegans)* which totals over 100 million base pairs, is an valuable tool we can use to more clearly understand the functions of its gene products (Hillier, Coulson et al. 2005; Schwarz 2005). This sequence lends itself to the formation of approximately 22,227 protein-coding genes, many of which have mutant alleles, providing us with a picture of at least the majority of the "players" involved (Spieth 2006). Many of these genes that have been sequenced have ancient origins, and are conserved in the domain eukarya, meaning they must be critical to properly function in the worm and animals in general. This is truly an incredible platform from which we can build and begin to truly understand the functions and mechanisms and the implications it means for metazoans (Hillier, Coulson et al. 2005; Schwarz 2005). Specifically in this study we aim to understated more clearly the functions and mechanisms of various genes after exposure to doses of nicotine by studying changes in gene expression.

Introduction:

When Sydney Brenner selected *C. elegans* in 1965 for his studies of organism development and the nervous system it was because of its simple anatomy, and the ease of genetic manipulation (Brenner 1974; Hillier, Coulson et al. 2005). The problem he sought to solve was how genes might dictate complex structures and regulatory networks found in higher organisms (Brenner 1974). The search for a multicellular organism that could be maintained and analyzed with ease resulted in a clear identification of *C. elegans* as a solution based on its

characteristics (Kenyon 1988). This approach has often proved successful for example, using the available *C. elegans* genome protein sequences (proteome) as a scaffold for identifying novel human genes, many biomedical researchers have been able characterize the function of many genes in the human proteome (Lai, Chou et al. 2000; Kaletta and Hengartner 2006).

C. elegans is an excellent model organism for research on this specific type of question. Genomic studies that compare genes, such as that of humans and *C. elegans,* offer a vast variety of candidates for the identification and functional mechanisms of human orthologous genes. In the *C. elegans* genome at least 83% (15,344 sequences) of the total 18,452 available *C. elegans* proteomes have human homologs. That is combined with the fact that approximately 7,954 records of *C. elegans* proteins have matching human gene transcripts (Lai, Chou et al. 2000; Hillier, Coulson et al. 2005). That means that *C. elegans* is a great models for determining pathways and functions of many different genes.

Protein-coding genes are genes that encode polypeptide chains that fold into proteins (Spieth 2006). This is the largest class of any of the gene types in *C. elegans,* and is considered to be the workhorse for the cell due to their numerous functions. Approximately 25.5% of the genome appears to be protein-coding in *C. elegans* (Hodgkin 2005). Protein-coding genes maintain a variety of functions within most organisms. Variation in the regulation of these protein-coding genes and their functions has been shown to affect many processes and characteristics in the host organism (Andrew K. Jones 2003; Feng, Li et al. 2006).

In this study we aim to look at the expression patterns of several protein-coding genes in response to nicotine, looking at different gene families to get an idea of effects of at the genomic level. We will the use this information to search for possible targets that may be regulated by

miRNAs. Genes with the highest and lowest expression as compared to nicotine untreated animals will be considered viable gene targets for miRNAs. We then can analyze the potential targeting miRNA expression pattern.

Materials and methods:

Synchronization

In order to extract enough total rna sample C. elegans were densely grown in 100x15 mm Petri dishes on nematode growth medium (NGM) and fed OP50 strain *Escherichia coli* according to a standard protocol (Hitchcock 1998; Brenner 2000), with Laval stage 3 from an age-synchronized culture used in all the experiments. To produce age-synchronized cultures, at 2–3 days, eggs from mature adults were isolated using a synchronization solution, followed by rinsing with M9 buffer (Hitchcock 1998). After rinsing worms were placed on an NGM plate with no food for an arresting period to ensure all worm were closely synchronized.

The worms were then transferred to NGM agar plates with a food source, resulting in synchronized adult worm populations. L3 larval stage worms were washed out using M9, rinsed, and then centrifuged at 2000 rpm for 2 min at room temperature a 2uL sample was taken and placed on a slide and counted under an inverse microscope to estimate the number of worms.

Preparation of Nicotine Treatment

A stock of 1,000 ppm nicotine was made from ACROSS organics L-nicotine, 98% pure (CAS 54-11-5) product. This was made by combining 10uL of L-nicotine with 9,990uL of distilled water. Five doses were chosen to test: control (0ppm), 1ppm 3.16ppm, 10ppm, 31.6ppm.

Doses were prepared to a volume of 20mL to accommodate the large amount of worms and ensure that the worms were equally exposed.

C. elegans Nicotine Treatment

Briefly, each test consisted of four concentrations and a control, in which the L3 stage worms from one densely grown 100x 15 mm nematode growth medium (NGM) petri dish, were transferred to a 50mL Falcon polystyrene conical tube containing nicotine dose. Each was made in 20 mL quantities from the 1,000ppm stock. These tubes were taped and placed in a rotator at 42 rpm in an incubator at 20^0C. The worms were exposed to the nicotine doses for 24 hours. After the 24 hr dosing period worms were collected and rinsed with M9 multiple times to remove any extra nicotine solution. Once rinsed, the worm pellet was transferred to a 1.5 mL microcenterfuge tube and all excess fluids were removed. The tubes were flash frozen in liquid nitrogen and then stored in a -80^0C freezer until ready for RNA extraction.

Total RNA Extraction

Total RNA was isolated from the dosed worms using the RNA/DNA/Protein Isolation Reagent from Applied Biosystems (Ambion, Austin, TX, USA) according to the manufacturer's protocol. Total RNA was then quantified and assessed for quality using a Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE, USA). After quality was assessed, RNA samples were stored at -80°C until further analysis.

RT-PCR and qRT-PCR for gene expressions

Applied Biosystems TaqMan microRNA Assays were employed to detect and quantify *C. elegans* protein-coding genes and microRNA using real-time PCR according to the

manufacturer's instructions. There were two steps in the TaqMan miRNA Assays: a) reverse transcription of the mature mRNA to a longer single-stranded cDNA sequence using a miRNA-specific stem-looped primer, and b) quantitative real-time PCR. In short, a single-stranded cDNA was generated from 1 μg of the total RNA from the control, 1ppm, 3.16ppm, 10ppm, 31.6ppm nicotine exposed *C. elegans* tissue samples. Reverse transcription was reacted in 15 μL solution, which contained 500 ng of total RNAs, 1 mM each of dNTPs, 1 μL MultiScribe Reverse Transcriptase (50 U/μL), 1.5 μL 10X TR Buffer, 0.188 μL RNase Inhibitor, and 3 μL 5X Taqman RT primer. The reverse transcription reaction was performed with a Eppendorf Mastercycler Personal PCR machine (Westbury, NY) with the following temperature program: initial 16°C for 30 minutes followed by 42°C for 30 minutes; then, the reaction was held for 5 minutes at 85°C; finally held at 4°C until next analysis or stored at -20°C.

For the second step of the assay we performed the quantitative real time PCR (qRT-PCR) with a Poly T primer and a gene specific primer. Each reaction was performed in 20 μL solution, which contained 2 μL RT PCR product (10-fold dilution from RT PCR reaction), 10 μL Taqman 2X Universal PCR Master Mix (No SmpErase UNG), and 1 μL Taqman MicroRNA Assays 20X Real Time Primers. Nuclease free water was used to adjust the final volume to 20 μL. The temperature program for qRT-PCR reactions were 95°C for 10 minutes followed by 45 cycles of 95°C for 15 seconds (to denature DNA) and 60°C for 60 seconds (for primer annealing and extending).

All reactions had three replicates and each dose had three replicates. In qRT-PCR, Y45F10D was employed as an endogenous reference gene for normalizing qRT-PCR results. The results were analyzed using the ΔΔCt method.

Results:

We tested several protein-coding genes for changes in expression. These genes belong to various families with a wide range of functions. They include: *acr-16, acr-8, cat-4, egl-10, egl-19, egl-49, egl-47, egl-5, hlh-14, lev-1, lev-8, lin-39, ric-3, unc-103, unc-29, unc-43, unc-50, unc-68, sod-1, pink-1, oxi-1, age-1, and old-1*.

Table 4-1 shows the percentage of up/down-regulation for each tested gene. The highest up regulated gene was *hlh-14*, with an up regulation of 13,814% (138-fold up-regulated). Conversely the lowest down regulated gene was *old-1*, with a down regulation of 92% (12.5-fold down-regulated). Figure 4-1 is the graphical representation of the fold change expressed by each of the tested genes at various concentrations. The 3.16 ppm bar for *hlh-14* (138-fold up-regulated) was cut off in order to allow the other bars to be visible.

Discussion:

The “acr” families of genes are mainly associated with the alpha and non-alpha subunits of the nicotinic acetylcholine receptor (nAChR). The two candidates from this family that were tested are *acr-16* and *acr-8*. The protein-coding gene acr-16, which encodes a nicotinic AChR subunit in *C. elegans,* is a homolog to the vertebrate α7 sub-unit (Francis, Evans et al. 2005). It was also shown that this gene mediated the nicotine-evoked ACh responses of the worm (Francis, Evans et al. 2005). Alternatively, in a separate study of acr-8 showed that it had no effect on either the levamisole-sensitive/insensitive components of the muscle ACh after its

deletion. Therefore, it is possible to hypothesize that the acr-8 is a non-essential subunit of the nicotinic receptor class in the ventral body wall muscles of *C. elegans* (Touroutine, Fox et al. 2005). This may also explain the inverse relationship of the gene expression for these two protein-coding genes we observed in our assays

The protein coding genes *acr-8* and *acr-16* display an inverse relationship to each other; while one was being up-regulated the other was down-regulated. Since both acr-8 and acr-16 are levamisole receptors in the muscle, they may be expressed differently due to the fact that acr-16 is more important for cholinergic synaptic currents (Touroutine, Fox et al. 2005). Also the only dose at which each gene was unexpressed among all four doses was at the concentration of 3.16 ppm, which may indicate regulation by gene expression is more sensitive at a specific dose of nicotine exposure. Table 4-2 gives a detailed explanation of the function of each of the specific genes used for this study.

The "unc" family of genes deals with many facets of gene expression including locomotion and again the nicotinic acetylcholine receptor (nAChR). The list of candidates used from this family include: *unc-103, unc-29, unc-43, unc-50, and unc-68*. Gene deletions give an idea of the functions of these genes. For example, with the unc-103 gene deleted, hermaphrodites prematurely laid embryos that would normally be retained in the uterus for full development; this may indicate that the gene *unc-103* is involved with egg-laying timing and muscle contraction (Reiner, Weinshenker et al. 2006). In our study this gene was down regulated at three ppm dosed concentrations possibly indicating that the exposure to nicotine causes this gene to be suppressed and caused an increase in premature egg laying. The up regulation of the "unc" genes at 10ppm may indicate a compensating mechanism exists when exposed to specific dose, basically an effort on the cells part to maintain normal egg-laying abilities. At higher concentrations the

expression of this gene is significantly down regulated possibly indicating cells have shifted from attempting to maintain normal function to a death cascade.

The *unc-29* gene has been shown to encode components of a nicotinic receptor that can promote egg-laying, but is not necessary for the muscle contractions needed for egg-laying (Kim, Poole et al. 2001). Our study shows that this gene was generally down regulated for the three tested concentrations and only slightly up regulated again at 3.16 ppm. This may indicate that after exposure to nicotine, this gene is suppressed and normal egg-laying promotion is decreased.

The gene *unc-50* has a homolog in humans called UNCL that is highly concentrated in the brain and is well conserved across many species (Fitzgerald, Kennedy et al. 2000). The gene *unc-50* does not encode a nAChR subunit but appears to be required for proper nAChR assembly (Table 5-2). In this study *unc-50* was up-regulated at all low doses except the highest dose, 31.6 ppm, where it was slightly down regulated. This may indicate that upon exposure to nicotine, more receptors need to be assembled for binding of the large influx of nicotine.

As seen in Table 4-1 and Figure 4-1, all the tested "unc" family genes except *unc-43* were up-regulated considerably at the concentration of 3.16 ppm. This again suggests that most nAChR related genes are turned on at certain exposure conditions. This is likely due to the fact that the nicotinic acetylcholine receptors (nAChRs) are pentameric membrane proteins that mediate excitatory synaptic transmission at neuromuscular junctions (Mongan, Jones et al. 2002). Nicotine is a known substrate of these proteins so it may stimulate the expression of the receptors (Lam, Girard et al. 2007).

The "cat" families of genes are mainly associated with catalase, a key antioxidant enzyme in the defense against oxidative stress. This gene is mainly down-regulated and only up-regulated

1.1 fold at a concentration of 10ppm. This suggests that under current exposure conditions, there is no significant oxidative stress.

The "egl" families of genes are mainly associated with the formation of transcription factors and seven transmembrane G-protein-coupled receptors. We used several candidates from this family including: *egl-10, egl-19, egl-49, egl-47, and egl-5*. The *egl-10* and *egl-49* exhibit 3 fold and 5.6 fold up-regulation at 1ppm, respectively, which are the genes most up-regulated at this low concentration. This indicates that egl-10 and egl-49 maybe "first response" genes to nicotine exposure.

Many behaviors that are regulated by genes can be measured by performing behavioral assays. One study suggests that the egg-laying behavior in *C. elegans* is regulated in a nicotine dose-dependent manner by the gene egl-10 (Koelle and Horvitz 1996). It was proposed that egl-10 may regulate G-protein signaling in the neural processes, since the egg-laying behaviors were dramatically affected in a loss-of-function egl-10 mutation. In our study, the gene egl-10 was highly up-regulated in all doses except at the concentration of 31.6 ppm, which was slightly down-regulated. This may reinforce that idea that in order to maintain proper egg-laying ability, egl-10 must be up-regulated to overcome the nicotine toxicity.

Studies have shown that egl-19 may play an important role in the regulation of depolarization-induced calcium influx in *C. elegans* neurons (Laine, Frokjaer-Jensen et al.; Frokjaer-Jensen, Kindt et al. 2006). Loss of function mutants of this gene have been shown to lose the response to gentle touch stimulations, but are still able to move (Frokjaer-Jensen, Kindt et al. 2006). In our study there was a trend of down regulation by this gene as the concentration increases. This may contribute to the loss of normal movement that we observed at higher concentrations of nicotine.

In humans there is an abundance of the neural G-protein Gαo, whereas in *C. elegans* neurotransmission by this Gαo protein (*egl-47*) inhibits egg-laying behavior and could also serve as a model for studying signaling of G-protein (Moresco and Koelle 2004). In many of the doses that *C. elegans* were exposed to, this gene was down regulated, indicating egg-laying behavior would remain relatively normal or even increase. At 3.16 ppm this gene was up regulated meaning egg-laying should decrease, this indicates the involvement of this gene in complex signaling pathways when the gene regulation is the most active in this dose.

The "*hlh*" families of genes are mainly associated with normal egg-laying, movement, body morphology, and larval development; more specifically, *hlh-14* is required for proper neuronal development, particularly for regulation of the asymmetric cell division. The candidate from this family was *hlh-14*. Among all tested genes, *hlh-14* had the highest up-regulated expression of more than 139 fold as compared to control at the concentration of 3.16 ppm (Table 4-1 and Figure 4-1). This may indicate that upon exposure to nicotine these protein-coding genes must be up-regulated to maintain the proper development and function of the neurons.

The "*lev*" families of genes are also associated with alpha and non-alpha subunits of the nicotinic acetylcholine receptor (nAChR). The candidates *lev-8*(alpha), *lev-1*(non-alpha) were generally up-regulated. *lev-1* is required for the normal locomotion and egg-laying behaviors, whereas lev-8 is required for normal rates of pharyngeal pumping and for fully wild-type responses (egg laying and body wall muscle contraction). A higher expression pattern is an indication that in the presence of the toxicant nicotine, these genes are required more to maintain normal functions lev-8 belongs to a nematode specific class of subunits and has been shown to be expressed in neurons, muscle cells, and epithelial-derived socket cells. *C. elegans* currently has the largest known nAChR subunit family of 27 nAChR subunits that have been identified

compared to just 16 in humans (Towers, Edwards et al. 2005). In our studies, *lev-8* was up regulated at all concentrations and may indicate its importance in maintaining normal functioning of neurons and muscle cells upon exposure to a neurotoxicant such as nicotine.

The last few gene families deal with oxidative stress. The *sod-1* associated with the copper/zinc superoxide dismutase was down-regulated in all concentrations. This could be explained by unknown transcriptional feedback control mechanisms (Yanase, Onodera et al. 2009). Studies also suggest that *sod-1* and *cat-4* do not play a role in the adaptive response against oxidative stress, so these genes remained down regulated under the tested exposure conditions (Yanase, Yasuda et al. 2002). The oxi-1 was up regulated at all concentrations and shows remarkable up-regulated expression of more than 102 fold at the concentration of 3.16ppm. This indicates that there was increased oxidative stress associated with nicotine exposure. The *old-1* is associated with a transmembrane tyrosine protein kinase (formerly TKR-1),The complete up regulation of this gene at all doses might indicate that there may be increased need in phosphorylation of proteins, in order to activate/inactivate them, at this concentration thereby requiring an increased activity of this kinase (Murakami and Johnson 2001).

Conclusion:

In this study, we dosed *C. elegans* with various concentrations of nicotine and analyse changes in the expression of 23 selected protein coding genes. We were able to quantitatively determine the expression pattern changes in the transcription level of these genes across the dose range. We were able to identify some nicotine sensitive genes which are up-regulated at low nicotine concentrations. We also concluded that the expression of individual genes are dose specific, possibly indicating a complex gene regulation network exists in response to nicotine exposure.

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Table 4-1. Percentage of up/down-regulation for each tested genes.

	1ppm	3.16ppm	10 ppm	31.6ppm
acr-16	-43%	255%	-36%	-47%
acr-8	53%	-69%	73%	107%
cat-4	-10%	-26%	9%	-16%
egl-10	200%	2170%	66%	-14%
egl-19	108%	10%	-14%	-7%
egl-49	460%	26%	490%	192%
egl-47	-66%	416%	-31%	-88%
egl-5	-78%	257%	-2%	10%
hlh-14	-33%	13814%	63%	21%
lev-1	11%	30%	-59%	9%
lev-8	56%	115%	25%	12%
lin-39	-72%	230%	-74%	5%
ric-3	1%	829%	-12%	-38%
unc-103	-76%	1107%	-85%	-68%
unc-29	-48%	116%	-36%	-25%
unc-43	36%	-43%	132%	-13%
unc-50	64%	983%	38%	-8%
unc-68	-33%	1163%	30%	151%
sod-1	-35%	-71%	-28%	-30%
pink-1	-1%	26%	19%	12%
oxi-1	44%	1014%	186%	22%
age-1	12%	475%	75%	-14%
old-1	-85%	105%	-92%	-60%

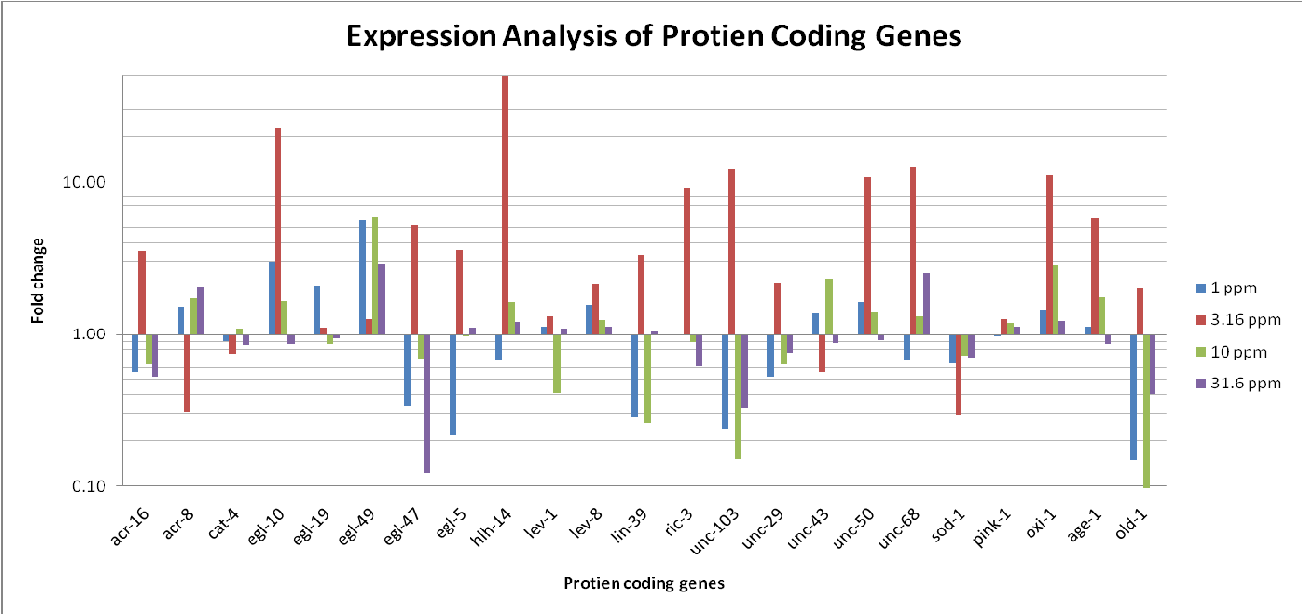

Figure 4-1. Protein coding gene expression vs fold change. Here we have the expression analysis of the 23 protein coding genes selected for this study. The fold change was calculated using the d-dct method of qrt-pcr analysis

Table 4-2. List of selected genes with functional descriptions (per *C. elegans* database)

Gene	Function
acr-16	acr-16 encodes an alpha-7-like homomer-forming subunit of the nicotinic acetylcholine receptor (nAChR) superfamily orthologous to human nicotinic cholinergic receptor alpha 7 (CHRNA7; OMIM:118511; possibly associated with schizophrenia and juvenile myoclonic epilepsy); ACR-16 functions as a ligand-gated ion channel that is required for the major fast cholinergic excitatory current at C. elegans neuromuscular junctions; an ACR-16::GFP reporter fusion expressed in muscle cells localizes to the tips of muscle arms, specific regions of the muscle cell membrane that form synapses with neuronal processes; acr-16::gfp promoter fusions also reveal expression in a subset of neurons; when expressed in Xenopus ooctyes, ACR-16 is active as a homomeric receptor and responds robustly to acetylcholine.
acr-8	acr-8 encodes an alpha subunit of nicotinic acetylcholine receptor (nAChR); ACR-8 falls into the 'ACR-8' class of nAChR subunits, which includes ACR-12 and ACR-13.
cat-4	cat-4 encodes an ortholog of the human GTP cyclohydrolase I gene (GCH1; OMIM:600225), which when mutated leads to dystonia; CAT-4 is involved in serotonin and dopamine biosynthesis that affects movement, mating behavior, foraging behavior, and cell migration; CAT-4 acts in the same genetic pathway as flp-1
egl-10	egl-10 encodes an RGS protein, expressed in neurons, that affects egg laying and negatively regulates GOA-1 (Galpha[o]) signalling; it requires the Gbeta(5) ortholog GPB-2 for this activity, and genetically interacts with the egl-30 and goa-1 signaling pathways
egl-19	egl-19 encodes an ortholog of the alpha subunit of mammalian L-type calcium ion channels that affects muscle contraction in late embryonic morphogenesis, movement, egg-laying, mating and feeding; egl-19 is expressed in muscle cells and some neurons
egl-49	egl-49 was identified in screens for genes required for development and function of the hermaphrodite specific motor neurons (HSNs) required for egg laying; mutations in egl-49 result in HSNs that are functionally defective but morphologically normal.
egl-47	egl-47 encodes two seven transmembrane G-protein-coupled receptors; while loss-of-function mutations in egl-47 result in no detectable egg-laying defects, gain-of-function mutations and overexpression studies suggest that

	EGL-47 is required for negatively regulating activity of the hermaphrodite-specific motor neurons (HSNs) that control egg laying; EGL-47::GFP reporters made with either of the two isoforms indicate that EGL-47 is expressed in a small subset of head neurons, the HSNs, and the PVQ tail interneurons; in addition, a reporter made with the longer isoform, EGL-47A, is expressed in vulval cells in L4 larvae; in adults, the only cells of the egg-laying system that express EGL-47 are the HSNs; genetic and overexpression studies suggest that, in the HSNs, EGL-47 signals through the G-alpha protein GOA-1 to inhibit egg laying.
hlh-14	hlh-14 encodes a basic helix-loop-helix (bHLH) transcription factor that is one of five C. elegans Achaete-Scute family homologs; HLH-14 activity is required generally for normal egg-laying, movement, body morphology, and larval development; more specifically, HLH-14 is required for proper neuronal development, particularly for regulation of the asymmetric cell division that gives rise to the PVQ/HSN/PHB neuroblasts and for normal differentiation of these neuroblast descendants, including their cell division patterns, migrations, and neurotransmitter expression; in specifying neuronal cell fates, genetic evidence suggests that hlh-14 acts together with hlh-2, which encodes the C. elegans E/Daughterless ortholog; HLH-14 is expresed exclusively in the embryo and detected in the left and right PVQ/HSN/PHB neuroblasts and their descendants, as well as in other cells identified, tentatively, as anterior neuroblasts and posterior neuroblasts such as Caapa.
egl-47	egl-47 encodes two seven transmembrane G-protein-coupled receptors; while loss-of-function mutations in egl-47 result in no detectable egg-laying defects, gain-of-function mutations and overexpression studies suggest that EGL-47 is required for negatively regulating activity of the hermaphrodite-specific motor neurons (HSNs) that control egg laying; EGL-47::GFP reporters made with either of the two isoforms indicate that EGL-47 is expressed in a small subset of head neurons, the HSNs, and the PVQ tail interneurons; in addition, a reporter made with the longer isoform, EGL-47A, is expressed in vulval cells in L4 larvae; in adults, the only cells of the egg-laying system that express EGL-47 are the HSNs; genetic and overexpression studies suggest that, in the HSNs, EGL-47 signals through the G-alpha protein GOA-1 to inhibit egg laying.
lev-1	lev-1 encodes a non-alpha subunit of nicotinic acetylcholine receptor (nAChR) which, when mutated, confers resistance to levamisole; LEV-1 is required for completely normal locomotion, regulation of egg-laying behavior, and forms a cation channel when coexpressed with UNC-38 or

	UNC-63 and UNC-29; LEV-1 falls into the 'UNC-29' class of nAChR subunits, which includes UNC-29, ACR-2, and ACR-3; a LEV-1::GFP fusion protein is expressed in all body wall muscle cells and a subset of ventral cord motor neurons.
lev-8	lev-8 encodes a novel nicotinic acetylcholine receptor (nAChR) alpha subunit that is a member of the ACR-8 group of nAChR subunits; LEV-8 activity is required for normal rates of pharyngeal pumping and for fully wild-type responses (increased egg laying and body wall muscle contraction) to the nAChR agonist and antihelmintic levamisole; expression of a LEV-8::GFP reporter construct begins at the L1 larval stage and is detected in neurons, body wall and uterine muscle cells, and socket cells of the IL and OL mechanosensory neurons; expression in body wall muscles is strongest in the anterior, consistent with increased levamisole resistance of head, or anterior, muscles seen in lev-8 mutant animals
lin-39	encodes a homeodomain protein homologous to the Deformed and Sex combs reduced family of homeodomain proteins; lin-39 is required cell autonomously for specification of mid-body region cell fates, including those of the VC neurons and the vulval precursor cells (VPCs), during postembryonic development; lin-39 activity is also required for normal migration of the QR, and to a lesser extent QL, neuroblasts and their descendants; in regions of the body where lin-39 expression overlaps with that of mab-5, another C. elegans HOM-C gene, the two genes appear to either compensate for one another's activity or act combinatorially to promote cell fates distinct from those where either gene is expressed alone; lin-39 transcripts are detected at all development stages, and a lin-39:lacZ reporter fusion is expressed in cells of the central body region, including ventral cord neurons and ventral epidermal cells, from mid-embryogenesis through larval and adult stages.
ric-3	encodes a novel, highly charged protein with two transmembrane domains and extensive coiled-coil domains; RIC-3 is necessary for the maturation and function of at least four nicotinic acetylcholine receptors; specifically, it is needed for assembly or trafficking of the DEG-3 nicotinic acetylcholine receptor; RIC-3 levels and hence, activity, are regulated by BATH-42, a BTB and MATH domain-containing protein, with which RIC-3 interacts in vitro; as BATH-42 also interacts with the CUL-3 cullin, RIC-3 levels are thus likely regulated by a CUL-3-containing ubiquitin ligase complex
unc-29	unc-29 encodes an non-alpha subunit of the nicotinic acetylcholine receptor (nAChR) superfamily; UNC-29 is required for normal locomotion and egg-

	laying, and functions as a subunit of a ligand-gated ion channel that likely mediates fast actions of acetylcholine at neuromuscular junctions and in the nervous system; when coexpressed with LEV-1, a non-alpha nAChR subunit, and UNC-38 or UNC-63, alpha AChR subunits, the resulting multimer can form levamisole-gated channels; UNC-29 is expressed in body wall muscle
unc-38	unc-38 encodes a nicotinic acetylcholine receptor (nAChR) alpha subunit; UNC-38 is required for normal locomotion and egg-laying, and functions as a subunit of a ligand-gated ion channel that likely mediates fast actions of acetylcholine at neuromuscular junctions and in the nervous system; when coexpressed with ACR-2, ACR-3, UNC-29, and LEV-1, non-alpha nAChR subunits, the resulting multimer can form levamisole-gated channels; UNC-38 is expressed postsynaptically in muscles and neurons where it colocalizes with TAX-6, and with ACR-8, ACR-12, and UNC-29, respectively
unc-50	unc-50 encodes an integral membrane protein orthologous to the Saccharomyces cerevisiae Golgi component Gmh1p; UNC-50 is required for regulating subtype-specific nicotinic receptor trafficking to the cell surface and thus, for normal synaptic transmission at the neuromuscular junction; UNC-50 is ubiquitously expressed from early embryogenesis to adulthood and localizes to the Golgi
unc-43	unc-43 encodes the C. elegans ortholog of type II calcium/calmodulin-dependent protein kinase (CaMKII); unc-43 activity is required for a number of processes, including locomotion, neuronal cell fate specification and regulation of synaptic density, egg laying, defecation, and meiotic maturation; in regulating some of these processes, unc-43 acts in concert with MAPKKK and G protein signaling pathways; UNC-43 is expressed in neurons, oocytes, and gonadal sheath cells; UNC-43 can regulate the activity of the EGL-2 ether-a-go-go potassium channel with which it physically interacts.
unc-68	unc-68 encodes a ryanodine receptor ortholog that is expressed in body-wall muscle cells and is required for normal body tension and locomotion; unc-68 mutants are flaccid, sluggish, and resistant to ryanodine, but have normal muscle ultrastructure; UNC-68 is dispensable for excitation-contraction coupling itself, but may amplify its calcium signals; the unc-68/kra-1(kh30) mutation is an S1444N substitution at a putative protein kinase C phosphorylation site; UNC-68 reduced unc-103(sy557)-induced spicule protraction by one half; unc-68 is orthologous to the human genes RYR1 (OMIM:180901, mutated in malignant hyperthermia and central core

	disease), RYR2 (OMIM:180902, mutated in stress-induced polymorphic ventricular tachycardia), and RYR3 (OMIM:180903)
unc-103	unc-103 encodes an ERG-like K+ channel homolog, orthologous to human KCNH6 (OMIM:608168); UNC-103 regulates muscle activation in motility, egg-laying and male spicule protraction; UNC-103 is expressed highly in many neurons; gain-of-function alleles can be uncoordinated, while loss-of-function alleles have a more subtle defect in copulatory spicule protraction
sod-1	sod-1 encodes the copper/zinc superoxide dismustase, an enzyme that is known to protect cells from oxidative damage; superoxide dismutase activity can be detected in worm extracts; sod-1 activity has been implicated in the increased life-span of dauer larvae where this enzyme demonstrates the highest activity compared to other life-stages as well as in the increased life span of age-1 mutants and their resistance to oxidative damage; sod-1 modulates the effect of let-60 ras on vulval and germline development via cytoplasmic reactive oxygen species; unlike other eukaryotic superoxide dismutases, sod-1 does not require the copper chaperone CCS for its activity and instead uses a glutathione pathway for acquiring copper; in humans, mutation of SOD1 (OMIM:147450) leads to amyotrophic lateral sclerosis (OMIM:105400)
pink-1	pink-1 encodes a predicted serine/threonine protein kinase that is most similar to the Drosophila and human PINK1 (PTEN-induced kinase) protein kinases, the latter of which has been implicated in familial forms of Parkinson's disease; although loss of C. elegans pink-1 activity via large-scale RNAi screens results in no obvious abnormalities, studies in Drosophila indicate that Drosophila PINK1 is a mitochondrial protein that genetically interacts with Drosophila parkin and is essential for maintaining mitochondrial function and integrity in several tissues, including flight muscles and dopaminergic neurons
age-1	age-1 encodes the C. elegans ortholog of the phosphoinositide 3-kinase (PI3K) p110 catalytic subunit; AGE-1, supplied maternally and embryonically, is a central component of the C. elegans insulin-like signaling pathway, lying downstream of the DAF-2/insulin receptor and upstream of both the PDK-1 and AKT-1/AKT-2 kinases and the DAF-16 forkhead type transcription factor, whose negative regulation is the key output of the insulin signaling pathway; in accordance with its role in insulin signaling, AGE-1 activity is required for regulation of metabolism, life span, dauer formation, stress resistance, salt chemotaxis learning, fertility, and embryonic development; although the age-1 expression pattern has not yet

	been reported, ectopic expression studies indicate that pan-neuronal age-1 expression is sufficient to rescue life-span defects, while neuronal, intestinal, or muscle expression can partially rescue dauer formation, and neuronal or muscle expression can rescue metabolic defects
old-1	old-1 encodes a receptor protein tyrosine kinase; old-1 activity is required for stress resistance and regulation of adult lifespan: old-1 mRNA expression is upregulated in response to stress and in daf-2 and age-1 mutant backgrounds; likewise, overexpression of old-1 in wild-type animals extends lifespan and increases resistance to heat and UV irradiation; an OLD-1::GFP fusion protein is expressed in the anterior region of worms, in neuronal, hypodermal, and pharyngeal tissues, as well as in the proximal region of the male gonad; expression is visible in young adults and appears to increase as animals age and in response to heat, starvation, or UV irradiation.
oxi-1	oxidative stress Induced

CHAPTER 5: miRNA gene expression analysis

Abstract:

The effects of nicotine on protein coding genes and their targeting miRNAs have rarely been studied. In this study we aim to further explore the effects of nicotine exposure on the expression of several selected miRNA genes, this focus on miRNA gene expression will be performed through the use of qRT-PCR. The miRNAs that were selected were predicted in-silico through the use of two computational target prediction miRNA data bases, miRNA.org and miRNAmap, to target the protein-coding genes analyzed in the previous chapter, the four different miRNAs were: Cel-mir-70, Cel-mir-58, Cel-mir-790, and Cel-mir-253. Of the miRNAs tested, Cel-mir-70 was the only one that showed an inverse relationship, as expected, with a miRNA and its protein-coding gene target. All other miRNAs did not show this inverse relationship, indicating that either they may not be the real regulators of the genes we analyzed or the miRNAs are not playing a role. This reinforces the idea that miRNA regulation of protein-coding genes is a very fluid and dynamic process. Understanding these expression patterns of the miRNAs will lead to a better understanding of their functions in gene regulation.

Introduction:

In a multicellular organism, one way to understand gene function is to understand how the gene works: when, where, and under what conditions a gene is expressed (Hillier, Coulson et al. 2005) . The toxicological results that are obtained from research pertaining to *C. elegans* are very likely relevant to higher animals including humans (Bischof, Huffman et al. 2006). The ability to perform genetic screens of the entire genome of *C. elegans* allows for a deeper insight into the

action of toxins, such as nicotine. Seeing the effects of various toxins and how *C. elegans* genome responds could lead to a greater understanding of molecular and genetic mechanisms (Bischof, Huffman et al. 2006).

C. elegans is an excellent model organism for exploring the genetic basis of toxicant actions important to human life. Also, they are particularly useful for the study of the ecotoxicological relevance of chemically induced responses of genes (Roh, Lee et al. 2006; Roh, Park et al. 2009). The complete genome of *C. elegans* is known and is very information-rich. Approximately 25.5% of the genome appears to be protein-coding, while genes that include: introns, 5' and 3' untranslated regions and regulatory regions take up altogether at least 40 % of the whole genome (Hodgkin 2005; Jones, Davis et al. 2007). microRNAs (miRNAs) are a class of small (~21 nt) endogenous gene regulators. miRNAs are genomically-encoded, untranslated RNA molecules of approximately 20-25 nucleotides (nt) in length (Ambros; Ambros 2004; Bartel 2004; Baskerville and Bartel 2005). They negatively control gene expression at the post transcriptional level by binding to targeting messenger RNA (mRNAs) and inhibit translation of mRNAs via imprecise antisense base-pairing and act by one of two mechanisms: labeling and targeting mRNA for degradation, or by inhibiting mRNA translation (Ambros, Lee et al. 2003; Bartel 2004; Ambros and Chen 2007). They control diverse processes such as cell fate specification, apoptosis, developmental timing, diseases, and metabolism (Sulston and Brenner 1974). The *C. elegans* genome encodes many miRNAs (Bartel 2004; Ambros and Chen 2007; Miska, Alvarez-Saavedra et al. 2007). Many of the *C. elegans* miRNAs are highly evolutionarily conserved from flies to humans (Vella 2005). The first miRNA identified in *C. elegans* was lin-4, reported by Victor Ambros and colleagues in 1993 (Vella 2005). The *C. elegans* genome encodes hundreds of miRNAs. Current literature indicates that 175 miRNAs have been identified in *C.*

elegans so far (MIR-Base). Many of the *C. elegans* miRNAs are highly evolutionarily conserved from flies to humans (Lai, Chou et al. 2000; Narahashi, Fenster et al. 2000; Nass and Hamza 2007).

miRNA genes are normally found in the intergenic and intronic regions of the genome (Baskerville and Bartel 2005). The transcription of the miRNA genes is performed by RNA polymerase II resulting in primary miRNAs transcripts (pri-miRNA) (Roush and Slack 2009). Similarly to the protein-coding targets of RNA Pol II, pri-miRNAs are processed into pre-miRNAs in the nucleus by the microprocessor complex (Denli, Tops et al. 2004) . The premiRNAs are then exported to the cytoplasm by Exportin 5, where they are further processed by Dicer into mature miRNAs (Yi, Qin et al. 2003; Dempsey, Mackenzie et al. 2005).

Biogenesis of the *C. elegans* miRNA is a well-known process and occurs in a similar manner in vertebrates. After the transcription of a miRNA gene, nuclear cleavage of the pri-miRNA by the Drosha RNase III endonucleases occurs. This enzyme cuts both strands of the pri-miRNA near the stem loop and generates~60–70 nt stem-loop shaped miRNA precursor (pre-miRNA) (Sulston and Brenner 1974). This pre-miRNA is transported to the cytoplasm by the export receptor Exportin-5. The nuclear cut by Drosha defines one end of the mature miRNA and cytoplasmic cut by Dicer, also RNase III endonuclease, defines the opposite one. Dicer recognizes the pre-miRNA and cuts both of its strands at about two helical turns away from the base of the stem loop. Then the mature miRNA is associated with RNA-induced silencing complex (RISC) (Vella 2005). Once incorporated into a cytoplasmic RISC, the miRNA will specify cleavage if the miRNA has sufficient complementarity to the mRNA, or it will repress productive translation if the miRNA does not have sufficient complementarity. These miRNAs

down-regulate gene expression by interacting with partially complementary sequences in the 3' UTRs of their target genes (Sulston and Brenner 1974).

The mature single-stranded miRNA binds to messenger RNAs (mRNAs) to interfere with their translational processing. It is estimated that a very small amount of our DNA actually encode for these miRNA (~1%), but those miRNA regulate nearly one-third of our encoded reference genes. Over recent years there has been a flourish of different and exciting methods developed to ascertain the exact function and mechanics for these miRNAs. Various bioinformatics databases, tools, and algorithms have been developed to help predict the sequences of miRNAs and their target genes. These various tools used in combination with in silico approaches; provide new bioengineering approaches for fine-tuning gene regulation.

Investigations on miRNAs offer new and exciting opportunities for scientists with bioengineering. Manipulation and understanding of these miRNA activities can lead to the integrative understanding of the gene network regulation from the molecular to the systems levels. Clinical applications also abound such as drug design for, possible treatments of diseases and addiction in addition to diagnostic innovations (Sun, Julie Li et al. 2010). Computational and bioinformatics studies can help develop new ways to design miRNAs for the enhancement of these applications towards understanding addiction and miRNA’s involvement. Improved innovative algorithms and analysis methods are essential for the further advancement of this type of research field.

Materials and methods:

Synchronization

C. elegans were densely grown in 100x15 mm Petri dishes on nematode growth medium (NGM) and fed OP50 strain *Escherichia coli* according to a standard protocol (Hitchcock 1998; Brenner 2000), with Laval stage 3 from an age-synchronized culture used in all the experiments. To produce age-synchronized cultures, at 2–3 days, eggs from mature adults were isolated using a synchronization solution, followed by rinsing with M9 buffer (Hitchcock 1998). After rinsing worms were placed on an NGM plate with no food for an arresting period to ensure all worm were closely synchronized.

The worms were then transferred to agar plates with a food source, resulting in synchronized adult worm populations. L3 larval stage worms were washed out using M9, rinsed, and then centrifuged at 2000 rpm for 2 min at room temperature a 2uL sample was taken and placed on a slide and counted under an inverse microscope to estimate the number of worms.

Preparation of Nicotine Treatment

A stock of 1,000 ppm nicotine was made from ACROSS organics L-nicotine, 98% pure (CAS 54-11-5) product. This was made by combining 10uL of L-nicotine with 9,990uL of distilled water. Five doses were chosen to test: control (0ppm), 1ppm 3.16ppm, 10ppm, 31.6ppm. Doses were prepared to a volume of 20mL to accommodate the large amount of worms and ensure that the worms were equally exposed.

C. elegans Nicotine Treatment

Briefly, each test consisted of four concentrations and a control, in which the L3 stage worms from one densely grown 100x 15 mm nematode growth medium (NGM) petri dish, were transferred to a 50mL Falcon polystyrene conical tube containing nicotine dose. Each was made in 20 mL quantities from the 1,000ppm stock. These tubes were taped and placed in a rotator at 42 rpm in an incubator at 20^0C. The worms were exposed to the nicotine doses for 24 hours. After the 24 hr dosing period worms were collected and rinsed with M9 multiple times to remove any extra nicotine solution. Once rinsed, worm pellet was transferred to a 1.5 mL Microcenterfuge tube and all excess fluids were removed. The tubes were then flash frozen in liquid nitrogen and then stored in a -80^0C freezer until ready for RNA extraction.

Total RNA Extraction

Total RNA was isolated from the whole *C. elegans* homogenate using the RNA/DNA/Protein Isolation Reagent from Applied Biosystems (Ambion, Austin, TX, USA) according to the manufacturer's protocol. Total RNA was then quantified and assessed for quality using a Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE, USA). After quality was assessed and deemed of high enough quality, having a ratio of nucleic acids to protein (260/280) of between 2.0 and 2.2 and a ratio of nucleic acids to organic compounds in solution (phenol, trizol and others aromatics like these) (260/230) of 1.5 to 2.0 organic compounds in solution (phenol, trizol and others aromatics like these)., RNA samples were stored at -80°C until further analysis.

RT-PCR and qRT-PCR for MicroRNA gene expressions

In total, we investigated changes in the expression levels of four different miRNAs cel-miR-70, cel-miR-58, cel-miR-790, and cel-miR-253. The miRNAs that were selected were predicted in-silico through the use of two computational target prediction miRNA data bases, miRNA.org and miRNAmap, to be the targets of the protein-coding genes selected in the previous chapter. One housekeeping gene (Y45F10D) was used as reference gene to normalized expression values. Applied Biosystems TaqMan microRNA Assays were employed to detect and quantify *C. elegans* protein-coding genes and miRNA using real-time PCR according to the manufacturer's instructions. There were two steps in the TaqMan miRNA Assays: a) reverse transcription of the mature miRNA to a longer single-stranded cDNA sequence using a miRNA-specific stem-looped primer, and b) quantitative real-time PCR. In short, a single-stranded cDNA was generated from 1 μg of the total RNA from the control, 1ppm, 3.16ppm, 10ppm, 31.6ppm *C. elegans* tissue samples by reverse transcription using the Applied Biosystems RNA/DNA/Protein Isolation Reagent. Reverse transcription was reacted in 15 μL solution, which contained 500 ng of total RNAs, 1 mM each of dNTPs, 1 μL MultiScribe Reverse Transcriptase (50 U/μL), 1.5 μL 10X TR Buffer, 0.188 μL RNase Inhibitor, and 3 μL 5X Taqman RT primer. The reverse transcription reaction was performed with a Eppendorf Mastercycler Personal PCR machine (Westbury, NY) with the following temperature program: initial 16°C for 30 minutes followed by 42°C for 30 minutes; then, the reaction was held for 5 minutes at 85°C; finally held at 4°C until next analysis or stored at -20°C.

For the second step of the assay we performed the quantitative real time PCR (qRT-PCR) with a Poly T primer. Each reaction was performed in 20 μL solution, which contained 2 μL RT PCR product (10-fold dilution from RT PCR reaction), 10 μL Taqman 2X Universal PCR Master Mix (No SmpErase UNG), and 1 μL Taqman MicroRNA Assays 20X Real Time Primers.

Nuclease free water was used to adjust the final volume to 20 µL. The temperature program for qRT-PCR reactions were 95°C for 10 minutes followed by 45 cycles of 95°C for 15 seconds (to denature DNA) and 60°C for 60 seconds (for primer annealing and extending).

All reactions had three replicates and each dose had three replicates. In qRT-PCR, Y45F10D was employed as an endogenous reference gene for normalizing qRT-PCR results. The results were analyzed using the ΔΔCt method.

Selection of miRNAs

Currently, there are a large number of tools or algorithms that have been developed for computational target prediction. Although the principles of these tools or algorithms are mainly focused on complementarity between the seed region of the miRNA and the predicted target, there are quite differences on predicted targets using different tools or algorithms. Also, since few validated miRNA targets are known, it is hard to tell which tool or algorithm has the best performance.

In order to select for the mRNA (protein-coding genes selected) targets for our miRNAs, we used two online miRNA data bases including: miRNA.org and miRNAmap. These are online data bases of the predicted mRNA targets of many of the miRNAs found in several species including: *Homo sapiens, Drosophila melanogaster,* and *Caenorhabditis elegans.* We used two data bases to increase our confidence that the selected miRNA were in fact targets of the selected protein-coding genes. Once a list was compiled of the predicted miRNa targets for each database the final selections were based upon both databases revealing the same miRNA targets.

Results:

As shown in Figure 5-1, cel-miR-70 showed different expression levels at different concentrations: at concentration 1ppm it was shown to be down-regulated 14.3 fold, at concentration 3.16 ppm it was shown to be up-regulated 1.34 fold, at concentration 10 ppm again a down-regulation of 33.3 fold, finally at concentration 31.6 there was a down regulation of 100 fold, at which the expression was close to the threshold level. This miRNA was also predicted to be the target of another selected protein-coding gene, egl-10. The expression levels were very similar so they were combined in figure 5-2.

Cel-miR-58 also showed various expression levels at different concentrations: at concentration of 1ppm it was down-regulated of 14.3 fold, at concentration of 3.16 ppm it was up-regulated 4.57 fold, at concentration of 10 ppm again a down-regulation of 8.3 fold, finally at concentration of 31.6 ppm there was a down regulation 10 fold.

Similarly, the cel-miR-790 was only up regulated at concentration of 3.16 ppm 1.87 fold. At concentrations of 1ppm, 10ppm, and 31.6 ppm it was down regulated 9.1, 16.7, and 16.7 fold respectively.

Again the cel-miR-253 showed similar expression patterns as others; only at the concentration of 3.16ppm it was up-regulated 1.8 fold . At concentrations of 1ppm, 10ppm, and 31.6 ppm it was down regulated 10, 16.7, and 200 fold, respectively, where the expression was very low.

Fold- change results were then graphed alongside their protein coding gene targets in (Figure 5-2).

Discussion:

Recent studies have shown that the expression of miRNAs, an important class of gene regulators, can be altered when an organism is exposed to a toxicant. Studies similar to this one have been performed in various model organisms. In this study we investigated changes in expression levels of four miRNAs, selected because they were predicted targets of the protein coding genes that were shown to be highly up/down regulated after exposure to various concentrations of nicotine in *C. elegans*.

Using RT-PCR and qRT-PCR, we analyzed the expression patterns in four different miRNAs in L-3 synchronized *C. elegans* after exposure to various concentrations of nicotine. We found that all the four miRNAs were down regulated excepted at one tested concentration of 3.16ppm (19.5μM). This study may suggest that microRNAs may be induced and more active at specific low concentrations.

Cel-miR-70, a miRNA involved in regulating genes encoding the alpha subunit of nicotinic acetylcholine receptor (nAChR), was down-regulated after exposure to 1ppm, 10ppm, and 31.6ppm of nicotine. At 3.16 ppm (19.5μM) it was up-regulated. This expression pattern was reversely related to its potential targeting gene acr-8, which was down-regulated only at 3.16ppm and up regulated at 1ppm, 10ppm, and 31.6ppm of nicotine. The expression of miRNA and its potential gene targets should be inversely related. The Cel-miR-70 is likely a real target of acr-8/egl-10. This is an example of a miRNA that can directly regulate the expression of its target genes (Figure 5-2) (Bracht, Van Wynsberghe et al.).

Cel-miR-58, a miRNA that is involved with the controlling of the encoding of two seven transmembrane G-protein-coupled receptors, was the most up-regulated at the concentration of

3.16ppm (4.5fold) among all four tested miRNAs. Although it was predicted by the data bases that cel-mir-58 could potentially target egl-47, the data in this case suggests otherwise, due to the fact that the expression pattern of the protein-coding gene and the miRNA are not inversely related as seen (Figure 5-2). Another possibility is that there is another unchecked miRNA paralog that may also act on these protein-coding genes resulting in a combined up/down regulation. In humans, the gene that is homologous to this cel-mir-58 is hsa-miR-450b-3p (Ibanez-Ventoso, Vora et al. 2008).

Cel-miR-790, a miRNA that encodes an ERG-like K+ channel homolog, orthologous to human KCNH6, was down regulated at 1ppm, 10ppm, and 31.6ppm concentrations of nicotine.. It was shown to be the second most up-regulated at the concentration of 3.16 ppm, when compared to the other four miRNAs. Although the data base predictions showed that Cel-miR-790 potentially target unc-103, the expression pattern of unc-103 at each concentration seems not to be inversely related to the expression of Cel-miR-70, as seen in (Figure 5-2), as it would be if it were a real target. Again this has a possible explanation of several unchecked miRNAs having the same target. This particular miRNA has been shown to have at least seven other miRNAs homologs in *C. elegans* and four in humans (Ibanez-Ventoso, Vora et al. 2008). It is thought that some miRNA may act redundantly within family types that are homologs (Kaufman and Miska).

Cel-miR-253, a miRNA that regulates the copper/zinc superoxide dismutase, which is known to be induced under high oxidative stress (Chen, Shan et al. 2003; Guan, Yu et al. 2003; Yu, Nordberg et al. 2003), was found down-regulated in *C. elegans* after exposure to increasing concentrations of nicotine. As seen in the previous example with the other miRNAs, the change in expression point occurred at the 3.16 ppm dose. It is possible that cel-miR-253 is only induced

under high concentrations and at extremely high concentrations becomes inactive by some means.

There are some emerging studies that suggest that many miRNAs act redundantly with other miRNAs or other pathways, meaning it is possible for more than one miRNA to function to up/down-regulate a particular protein-coding gene (Miska, Alvarez-Saavedra et al. 2007). While fewer than 25% of protein-coding genes have a recognizable paralog in the *C. elegans* genome, about 60% of miRNAs are members of a family of two to eight genes

Conclusion:

The genetic analysis of miRNA genes in model organisms such as *C. elegans* is beginning to put into place the pieces of a mosaic that will eventually show us the range of functions that miRNAs have in the control of animal development and physiology (Ambros 2004). To assess the possible involvement and function of these miRNAs, studies like this one will continue to paint a picture of gene regulation and expression of miRNAs across all the phyla. This picture will eventually be able to answer questions like: Can miRNAs be activated and repress their targeting gene expression?

In this study we analyzed the expression of four miRNAs (cel-miR-70, cel-miR-58, cel-miR-790, cel-miR-253) selected after blasting to find potential miRNA targets of the selected protein-coding genes. The miRNAs were tested after exposure to various concentrations of nicotine including: control (0ppm), 1ppm, 3.16ppm, 10ppm, 31.6ppm (which corresponds to 6.17µM, 19.5µM, 61.7µM, and 194.5µM respectively). We found that individual miRNA expression profiles varied between the different concentrations, indicating that the nicotine concentration induces a differential miRNA expression.

When compared to the results of the protein-coding gene analysis, only one miRNA (Cel-miR-70) showed the inverse relationship expected if it were a true targeting miRNA. The rest of the miRNAs did not show this relationship, it can be concluded that maybe these are not true targeting miRNAs for the selected protein-coding genes

Therefore, we believe that miRNAs may play a key role in controlling protein-coding gene expression. Furthermore we believe that the understanding of this relationship between the toxicant (nicotine) and its effects on miRNAs will lead to a greater understanding and treatment of nicotine addiction. *C. elegans* is an appropriate model for studying nicotine's effects on not only miRNAs but protein-coding genes and the cytotoxicity as well. As mentioned before both humans and *C. elegans* share many pathways and mechanisms, any model that can help elucidate the various effects of different toxicants is a valuable tool for research.

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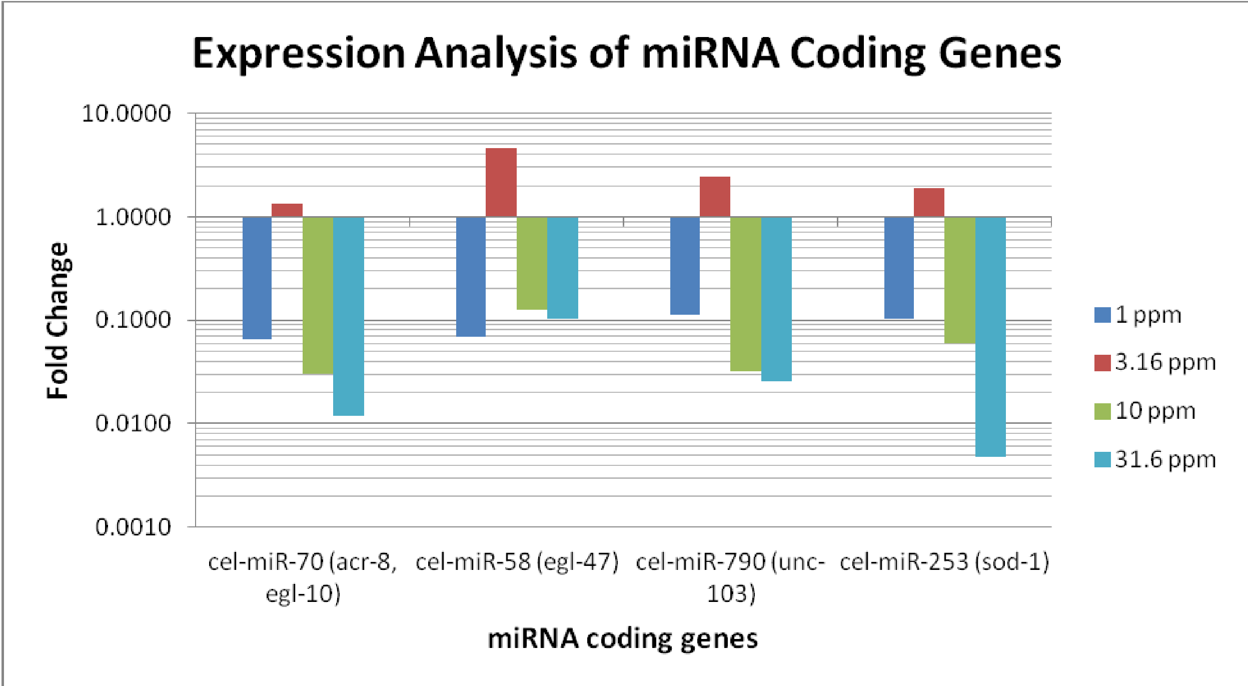

Figure 5-1. Gene expression analysis of four miRNAs after exposure to various concentrations (ppm) of nicotine. Results of the qrt-PCR were analyzed using the ΔΔCt method and fold changed was graphed

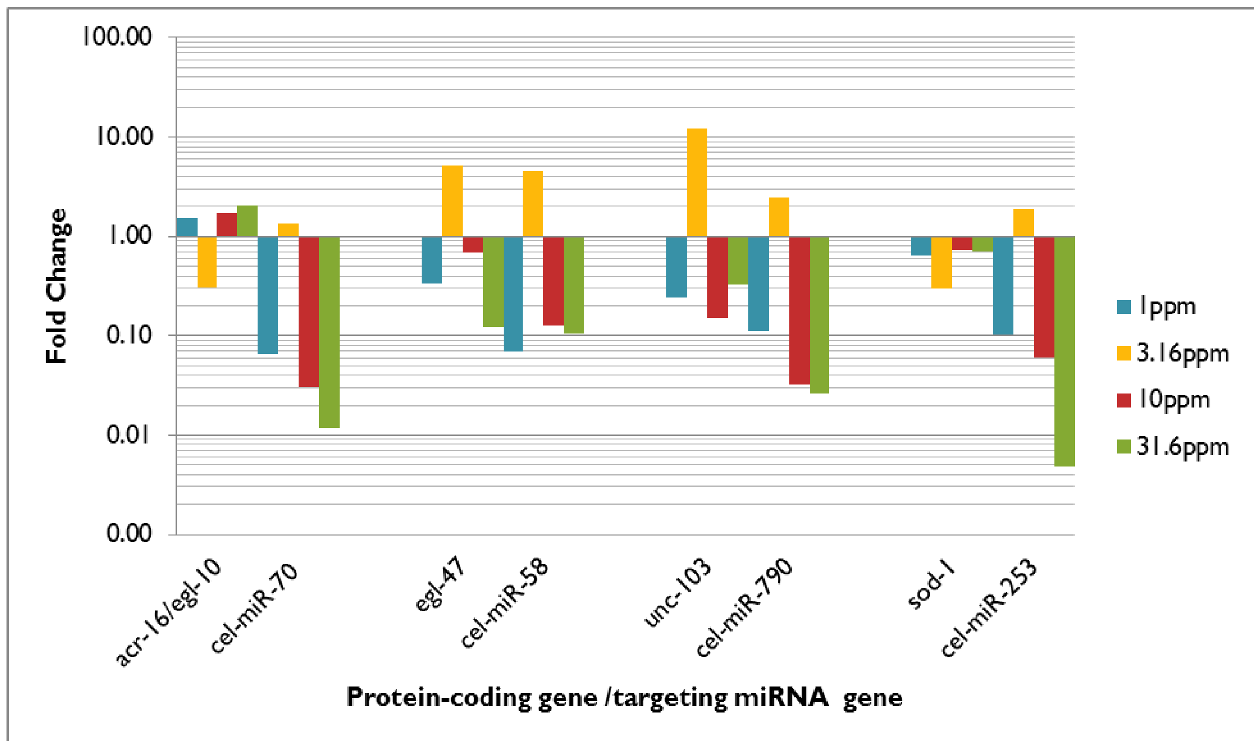

Figure 5-2. Gene expression analysis of the protein-coding gene target and the predicted targeting miRNAs after exposure to various concentrations (ppm) of nicotine. Results of the qrt-PCR were analyzed using the ΔΔCt method and fold changed was graphed