Vectors of transmission: An early nicotine puff triggers a long-lasting microRNA molecular "memory" in *C. elegans*.

by

Faten Taki

June, 2016

Director of Dissertation: Dr. Baohong Zhang

Department of Biology

The prevalence of passive and active nicotine exposure among neonates, children and adolescents remains relatively high and varies among cultures and countries. Currently, there is ample evidence that a safe nicotine dose or level does not exist. For that, early exposure to any nicotine product is considered "catastrophic" to the children's future and wellbeing. The cycle of nicotine addiction, relapse, and enduring health disparities remains unpreventable. This is majorly due to the incomplete understanding of nicotine-induced molecular signaling and mechanisms. Recently, the role of epigenetics in the developmental origin of diseases has been highlighted. With this in mind, this study aims to identify and validate the roles of particular epigenetics factors known as microRNAs in nicotine-induced early and late-onset diseases. Our workflow started with post-embryonic exposure to nicotine and was followed by global microRNA profiling across three generations. Based on the transcriptomic findings, we identified 14 transgenerational miRNAs that can serve as biomarkers for early parental nicotine exposure. Bioinformatics analysis coupled with a literature review allowed us to focus on three

factors: mpk-1, sir-2.1, miR-80 for further validation. We were intrigued in finding a potential relationship among these factors (MS80) in mediating nicotine induced early and late-onset diseases. For that, we performed reverse genetics assays and tested their contribution to nicotine-induced larval developmental delay, larval pharyngeal pumping inhibition, adult reproduction, adult mean lifespan, and adult germ line apoptosis. Our results showed that nicotine delayed development in an MS80 independent manner. Meanwhile, MS80 had a role in mediating nicotine-induced pharyngeal pumping inhibition. As for nicotine enduring effects, our data showed that post-embryonic nicotine exposure delayed the onset of reproduction without impacting fertility. This early exposure also had a negative effect on mean worm lifespan which was promoted by nicotine-induced upregulation of miR-80. In addition, our data also allowed us to infer that early nicotine exposure increases the ROS levels which trigger an organism level response that is mediated by MS80 to induce adult germ line apoptosis. Our study is the first to show the role of MS80 in intra and transgenerational inheritance of nicotine-induced effects. We also identified functional human homologs for cel-miR-80 in nicotine addiction and discussed the promising potential of manipulating MS80 to counteract nicotine-induced cellular and organismal damage during the early stages. Therefore, MS80 could be used as a prevention approach for nicotine-induced late-onset diseases.

Vectors of transmission: An early nicotine puff triggers a long-lasting microRNA molecular "memory" in *C. elegans*.

A Dissertation Presented to the Faculty of the Department of Biology

East Carolina University

In Partial Fulfillment of the Requirements for the Doctoral of Philosophy Degree in

Interdisciplinary Doctoral Program in Biological Sciences

by

Faten Taki

June, 2016

VECTORS OF TRANSMISSION: AN EARLY NICOTINE PUFF TRIGGERS A LONG LASTING MICRORNA MOLECULAR "MEMORY" IN *C. ELEGANS'*

by

FATEN TAKI

APPROVED BY:

DIRECTOR OF DISSERTATION: ----------

Baohong Zhang, PhD

COMMITTEE MEMBER: ----------

Xiaoping Pan, PhD

COMMITTEE MEMBER: ----------

Yong Zhu, PhD

COMMITTEE MEMBER: ----------

Myon Hee Lee, PhD

COMMITTEE MEMBER: ----------

Qing Cao, MD/CMD

CHAIR OF THE DEPARTMENT OF BIOLOGY: ----------

Jeff McKinnon, PhD

DEAN OF GRADUATE SCHOOL: ----------

Paul Gemperline, PhD

DEDICATION

I dedicate my dissertation to the most loving parents in the world, Ahmad and Lana Taki.

You are the bows from which your children
as living arrows are sent forth.
The archer sees the mark upon the path of the infinite,
and He bends you with His might
that His arrows may go swift and far.
Let your bending in the archer's hand be for gladness;
For even as He loves the arrow that flies,
so He loves also the bow that is stable.
(Gibran Khalil Gibran)

Thank you for being my stable bow.

ACKNOWLEDGEMENTS

My acknowledgements can extend to be a stand-alone chapter. This only proves how fortunate I was and am to be surrounded by a group of individuals who helped me in finishing my journey here in a myriad of ways.

To start, I would like to thank my Ph.D. mentor Dr. Baohong Zhang for believing in me. His prompt and very encouraging replies played a major role in taking the big leap from Beirut, Lebanon to Greenville, NC. Dr. Zhang always encouraged me to work hard and aim higher and he lead by example. He was not only my PhD mentor, but also a friend as he encouraged me to find a work life balance and to think about my future from a professional and as well as a family perspective. I owe my current and future success to him and I am forever grateful for the many opportunities and the avenues he pushed me to seek and accomplish.

Secondly, I would like to express my gratitude to Dr. Xiaoping Pan, Dr. Yong Zhu, Dr. Myon-Hee Lee, and Dr. Qing Cao for serving on my committee. They provided me with not only technical support, but also very meaningful advice and discussions on my health, future, and career. Most importantly, they instilled confidence in me especially in moments of self-doubt and provided the best feedback to finish my research and work and to plan my future step.

A special thank you goes to Dr. Yiping Qi, Dr. Tom Fink, Dr. Levi Lowder, Dr. Chao Li, Dr. Dong Yoon, Dr. Fuliang Xie and my colleague and friend Dongteng Liu for their great help and discussions. I learned so much from their experiences and really appreciated their time to troubleshoot and finish my experiments successfully. I am forever grateful.

I was also very lucky to have wonderful undergraduate mentees, Krishna Patel, Sana Bharde, and Robyn Alston, on our research team. I will never forget past and present members of the Zhang and Pan labs who became my family abroad and I am thrilled that we all get to graduate together.

My sincere thank you goes to our Biology Department and especially Dr. West, Dr. Stellwag, Dr. Scemama, Dr. Mckinnon, Ms. Joyce Beatty, and Ms. Jennifer Jacobs for the opportunity to teach and for making the experience a very pleasant and fruitful one and for their continuous help and support to participate in national conferences and networking events.

Last but not least, I would never have been able to come abroad if it were not for the best support system in the world, my family: my mom and dad who gave me more love than I thought existed in the world and supported my dreams from the beginning, even if it meant being the "worm girl"; my brother Ali and sister Nina for being my best friends and backbone; my aunts and uncles: Joumana and Suzana , and Salah and Ali for coming to my rescue whenever I doubt my decisions or problems arise.; my uncles Ahmad and Khodor for making me feel like the most special niece and for being my international role models everywhere they went and go; my grandmas Amira and Lutfiyyeh and aunts Nadia, Zeinab, and Khadijeh for praying for me always and for making me feel so special; my cousins, younger and older, for their warmest and sweetest words and thoughts. I am overwhelmed by their love which makes my journey both easier and harder wherever I go.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AAA	Abdominal aortic aneurysm
ACR (acr-7)	AcetylCholine Receptor
ADF	Serotonergic Sensory neuron
Ago	Argonaute
BBB	Blood Brain Barrier
C. elegans	Caenorhabditis elegans
cDNA	Complementary DNA
Ct	Threshold cycle (Ct) values
DA	Drugs of Abuse
DAG	Directed Acyclic Graph
DAVID	Database for Annotation, Visualization and Integrated Discovery
DGCR8	DiGeorge syndrome critical region gene 8
dNTP	Deoxyribonucleotide triphosphates
DTC	Distal Tip Cells
eIF4E	Eukaryotic translation initiation factor 4E
eIF6	Eukaryotic translation initiation factor 6
GFP	Green Fluorescence Protein
GOrilla	Gene Ontology enRIchment anaLysis and visuaLizAtion tool
GW182	Protein family with multiple glycine–tryptophan repeats (GW repeats)
HIV	Human Immunodeficiency Virus
MAPK	Mitogen Activated Protein Kinase
MeV	MultiExperiment Viewer
MFC	Mean Fold Change
mir/ miR	MicroRNA
NSM	Pharyngeal neurosecretory, motor, sensory neuron
OP50	Uracil auxotroph E. coli strain which has limited growth on NGM plates.
ORF	Open Reading Frame
qRT-PCR	Quantitative real time polymerase chain reaction
RFP	Red Fluorescence Protein
RISC	RNA-induced Silencing Complex
RNP	RNA-binding proteins
ROS	Reactive Oxygen Species
sir-2.1	Silent Information Regulator
SPSS	Statistical Package for the Social Sciences
SUD	Substance Use Disorder
UTR	Untranslated region
WHO	World Health Organization

LIST OF RECIPES

Media and food

NGM (Nematode Growth Medium) Agar	Total V (1L)
NaCl	3g
Peptone	2.5g
Agar	17g
Streptomycin (optional)	0.2g
Distilled water	975ml
Mix on shaker and autoclave at 121^{o}C for 20 min	
Wait for it to cool till about 55^{o}C (this is an important step before adding cholesterol)	
After cooling, add in the following order:	
Cholesterol (5mg/ml in ETOH)	1ml
CaCl2 (1M)	1ml
MgSO4 (1M)	1ml
Potassium Phosphate (PH 6)	25ml
Mix well (stir bar) and pour to the plates of interest using electronic pipette such that: 3.5 cm plate→ 3ml 6 cm plate→ 8-12 ml (depending on the petri dish brand) 10 cm plate→ 23-30 ml (depending on the petri dish brand)	
After solidifying and cooling, seal them and put them at RT overnight. Next day, check for any possible contamination. If all is clear, stack the dishes in their original bag and place in the 4^{o}C fridge for use later.	

Luria Broth (LB)	For V=1L
Tryptone	10g
Yeast extract	5g
NaCl	5g
dH_2O	Up to 1L
PH=7.5	Optional adjustment
Autoclave and cool to 55^{o}C before streptomycin (200mg) (Optional)	

LB medium (Solid)	For V=1L
Tryptone	10g
Yeast extract	5g
NaCl	10g
Agar	10g
dH_2O	Up to 1L
Autoclave	

OP50 Food stock for *C. elegans*
Prepare 300 ml LB liquid medium (see recipe #) and wait for it to cool after autoclave.
Inoculate with an OP50 pellet stored at -20^{o}C.
Place on a shaker (121 rpm) at 37^{o}C for 12-24 hours.

Transfer 10-12 ml of the grown bacterial solution to 25-30 Falcon tubes (15ml).
Centrifuge at 3000 rpm for 20 minutes.
Discard supernatant (usually just flip the tube and release liquid into the sink).
Dry the tip of the tube with a tissue paper.
Label the tubes, and store them at -20^{o}C.
To use the pellet, just add a certain number of M9 to it. It is better to measure the concentration of your bacteria before use. To do that, blank the spectrophotometer with M9. Add the same volume of the bacterial solution and measure the Absorbance (A) at λ=600nm. I usually have it b/t 1-2. For a 3.5 cm plate→15µl OP50 solution. For a 6 cm plate→ 50-60µl OP50 solution. For a 10 cm plate→120-150µl OP50 solution.

Buffers

$CaCl_2$ (1M)	For a V=100ml	
	$CaCl_2.2H_2O$	14.7g
	dH_2O	Up to 100ml
	Autoclave	
$MgSO_4$ (1M)	For a V=100ml	
	$MgSO_4.7H_2O$	24.6g
	dH_2O	Up to 100ml
	Autoclave	
Potassium Phosphate (PH 6)	For a V=1L	
	KH_2PO_4 (1M)	118.3g (869ml)
	K_2HPO_4 (1M)	23g (132ml)
	dH_2O	Up to1L
	Autoclave	
Cholesterol solution (5mg/ml)	Cholesterol	50mg
	Ethyl alcohol	10ml
	Do not autoclave	
M9 buffer	For V=1L	
	$Na_2HPO_4.7H_2O$	11.32g
	KH_2PO_4	3g
	NaCl	5g
	$MgSO_4.7H_2O$	0.25
	Sometimes M9 clouds+turns whitish. This is due to the MgSO4.7H20. I made a 100mM stock (M=246.4755g/mol). For 1L M9, I take 10.14 ml.	
	dH20	Up to 1L

Synchronization solution and protocol

1. Grow gravid worms (such that the worms are not so old and are laying fertilized eggs).
2. Wash each plate with 3-5ml of M9 until most worms are collected into a 15ml Falcon tube.
3. Centrifuge at 2000rpm for 2 min.
4. Discard supernatant and add 5ml of M9. Mix a bit.
5. Centrifuge at 2000rpm for 2 min.
6. Discard supernatant. (If the supernatant is still unclear, then repeat the wash).
7. Prepare a FRESH synchronization solution (with bleach added in the end). The recipe is as follows

For V=25 ml	
Water	17.5ml
NaOH	2.5ml
Clorox bleach	5ml
Note: If the bleach is scented, it might negatively affect the hatching of the eggs. In our lab, it seems that the bleach with the brand name (Top Job) is working for synchronization)	
Mix well	

8. For each 15ml Falcon tube, add 5ml of synchronization solution.
9. Mix vigorously by shaking/vortexing intermittently.
10. Check for the worm's status as you shake. Shake for 5 minutes (8 minutes maximum). The adult worms should break open and dissolve, leaving only free eggs dispersed in solution.
11. When ready, you have to work fast!
12. Quickly centrifuge the eggs in bleach at 2000rpm for 2 min at RT.
13. Remove supernatant.
14. Add 5ml of M9, and centrifuge for 2 min at 2000rpm.
15. Repeat this wash for 2 more times.
16. After removing the M9 from the third wash, add another 5ml of M9 to disperse the eggs. Note: at this point, I usually smell the tube to see if there is still bleach in it. If so, I repeat the wash.
17. Place the tube on a shaker (for aeration) overnight (12-18 hours) for the eggs to hatch to L1.
18. Since there is no food in the tube, the larvae will be stuck at L1 no matter what time the eggs hatched. Thus the worms are synchronized.
19. Next morning, centrifuge the tube, remove supernatant leaving about 100µl.
20. Transfer the larvae to an NGM plate already seeded with OP50.

Chapter I

MicroRNAs and nicotine: a double-edge sword

Abstract

Nicotine was first discovered in the 1560s and was introduced as a panacea. Unfortunately, increasing scientific evidence in the following decades proved that it is far from a miraculous drug as it harms every organ in the body and currently kills more than 480,000 individuals annually. Currently, there are no safe therapeutics for nicotine-related diseases among children and adolescents and this is partly because the molecular mechanisms for nicotine's effects in different developmental stages remain unknown. Having said that, the roles of epigenetics factors like non-coding RNA in substance use disorders has increased tremendously in the last few years. We were interested in investigating the roles of one RNA species known as microRNAs in response to early nicotine exposure. We reviewed the miRNAs response to a variety of abused drugs and found global differential expressions across different studies on nicotine. We were intrigued to validate the roles of nicotine-responsive microRNAs in response to early nicotine exposure. For that, we employed the model organism *C. elegans* to perform our profiling and reverse genetics experiments. We took advantage of its life history traits concerned with the short generation time and transparent body as well as the high genome sequence homology with humans. We performed systemic miRNA profiling and used pharyngeal pumping and adult germ line apoptosis as proxies for early and late-onset diseases. In this chapter, we provided background information on the conservation of these physiological phenotypes to mammalian systems which allows for the extrapolations of our findings.

Keywords: nicotine, epigenetics, microRNAs, C. elegans, MAPK, sirtuins, pharyngeal pumping, heart, cardiovascular, germ line apoptosis, neurodegenerative

Introduction

First line therapies for tobacco smoking cessation are nicotine-based. This only mitigates risks from other harmful chemicals in tobacco, but does not protect against nicotine addiction. Other non-nicotine based therapies like Bupropion (non-specific agent), Varenicline (nicotinic receptor partial agonist), Nortriptyline (affinity for multiple receptors), Clonidine (alpha2 - noradrenergic agonist) also improved cessation rate. Unfortunately, benefits are countered by side-effects ranging from nausea and insomnia to serious cardiovascular events and even death. Due to these side effects, no nicotine-free treatments are FDA-approved for children or adolescents (Karpinski, Timpe, & Lubsch, 2010). This reinforces the cycle of addiction and/or predisposition to future health burdens which stresses on the importance of novel approaches that are both safe and effective.

Addiction is complex arising from additive contribution of many genes (NIDA, 2014). This makes traditional gene therapy approaches far reaching. For that, epigenetic factors represent attractive candidates for treatment. We are particularly interested in microRNAs which are known to fine tune the expression of at least 50% of genes (Chekulaeva & Filipowicz, 2009). One miRNA can target hundreds of genes (Guo, Ingolia, Weissman, & Bartel, 2010), a property that can be exploited in polygenic disorders like addiction. Single as well as global miRNA expression profiling can be used in diagnostics as they represent physiological biomarkers. In addition, some miRNAs have become phase III medicines (e.g. NCT01200420 for hepatitis C) (Janssen et al., 2013). Here, we propose to identify a microRNA-dependent mechanism of nicotine-induced disorders.

Experiments using cell lines, though informative, often don't represent organism level responses. miRNAs and other signaling pathways can work in a non-cell-autonomous manner. On the other hand, gene manipulations coupled with imaging assays are often challenging in higher organisms. These challenges can be overcome by using the available genetics and behavioral tools in the model *C. elegans*. Using the worm, we will investigate the role a molecular network in mediating divergent and enduring impacts of early nicotine exposure.

First we identified nicotine-linked microRNA transgenerational biomarkers using qRT-PCR profiling. These microRNAs were used in bioinformatics analyses to predict gene targets and pathway enrichments (Chapter II). We integrated our bioinformatics predictions with a literature review and aimed to focus on three factors: mpk-1, sir-2.1, and miR-80 (Chapter III). Then, we employed reverse genetics to understand the role of those factors in nicotine-induced early (Chapter III) and late onset diseases (Chapter IV). Both larval growth rate and pharyngeal pumping rate were used as proxies for early onset phenotypes (Chapter III) while adult reproduction, lifespan, and germ line apoptosis were used as proxies for late onset endpoints (Chapter IV). Finally, we present our hypothesized model for nicotine-induced early and late-onset complications and provide evidence of potential functional homologs in humans (Chapter IV).

Significance

Our findings allowed the i) identification of a molecular mechanism for direct and enduring effects of postembryonic nicotine exposure (i.e. adolescent neuromotor dysfunction vs. adult germ-cell apoptosis), (ii) identification of microRNA targets for the development of

potentially safer therapeutics for early nicotine exposure, and (iii) the functional annotation of miR-80 in nicotine-SUD phenotype.

Background

Nicotine

Nicotine was named after the French ambassador Jean Nicot who introduced it to Queen Catherine de Medicis as a panacea in 1560 (Cotton, 1998) (Figure1.1). Centuries later, this 'panacea' kills more than 480,000 individuals annually (CDC, 2010a). According to World Health Organization (WHO), about half of the world's children (~700 million) are exposed to nicotine directly by breathing tobacco-polluted air (WHO, 2007) or indirectly through absorption/adsorption of third hand smoke residing on surfaces (Martins-Green et al., 2014). More so, more than 50% of teenagers are active smokers in a number of developing countries (WHO, 2013) and waterpipe tobacco smoking (i.e. hookah) has increased dramatically among youth in developed countries like the United States (Soule, Lipato, & Eissenberg, 2015). The very first published report on the dangers of tobacco "snuff" was in 1761 by Dr. J. Hill (J. Hill, 1761). Then, the first report on the adverse effects of environmental tobacco smoke on children's health in 1967(Cameron, 1967) started a snowball which continues to grow with more evidence on the sensitivity for tobacco during early human development. It was concluded that "there is no threshold dose of environmental tobacco smoking below which an effect will not occur" (Corbo et al., 1996; Potera, 2010). Among the 4000 chemicals that constitute tobacco smoke, nicotine is the most addictive and is notorious for being a poison that affects every organ in the body and is linked to a multitude of diseases (CDC, 1988, 2010a).

Nicotine pharmacology

Nicotine is a naturally occurring insecticide mainly found in tobacco leaves, but can also be found in small amounts in other common vegetables like eggplants and tomatoes (Domino , Hornbach , & Demana 1993) (Table 1.1). Nicotine has pKa of 8.0 and is considered a weak base. So, its absorption is dependent on the pH. For example, nicotine is ionized in acidic environments and is thus absorbed at a much slower rate through membranes. With this in mind, the routes of exposure are concomitant on the nicotine products. Typically, nicotine is ionized in cigarettes which are slightly acidic and thus does not get absorbed in the buccal cavity of active smokers. A slower route of absorption is through the skin and is the basis for transdermal patches and for the occupational hazard for tobacco harvesters. On the other hand, nicotine is readily absorbed by the buccal cavity of passive smokers and pulmonary tract of passive and active smokers, respectively since it is unionized in the released smoke (N. L. Benowitz, Hukkanen, & Jacob, 2009). From the alveoli, nicotine gets absorbed to the pulmonary venous circulation, enters the heart and then to the arterial circulation which delivers nicotine to the brain faster than intravenous injection. Like an excited child in a state fair, nicotine rides all the brain tissue by stereoselectively binding to nicotinic acetylcholine receptors (nAChRs). Those ligand gated channels open and result in an influx of cations like calcium and sodium and influence intracellular signaling (N. L. Benowitz et al., 2009; Grando, 2014) and consequently impact tissue function and organism physiology and psychology. Thus, nicotine impacts the brain before reaching the liver and once it does, it is primary metabolized by cytochrome CYP2A6 and then by other enzymes resulting in around 16 additional metabolites, some of which are alkylating agents. More so, nicotine can also permeate the cell membranes directly (Nielsen & Rassing, 2002) or via H+ gradient-dependent cation transport systems and then impact intracellular signaling (Kichko et al., 2013; Takami, Saito, Okuda, Takano, & Inui, 1998). With a half-life of

about 2 hours, nicotine also accumulates in different fluids such as saliva, gastric juice, fetal serum, amniotic fluid, and breast milk as well as body organs like the brain, blood, muscle, liver, kidney, spleen, and lungs. The arterial blood concentration of chronic smokers is about 100ng/ml and has been reported to be 10 folds higher in some situations (N. L. Benowitz et al., 2009).

Nicotine-related diseases

Nicotine is anorexic (H. Huang, Xu, & van den Pol, 2011; Mineur et al., 2011; Rubinstein & Low, 2011) and exposure during the perinatal period is associated with higher risks of sudden infant death syndrome (SID), low birth weight, decreased head circumference, as well as a developmental delay in genitals, skeletal muscle (Gyekis, Anthony, Foreman, Klein, & Vandenbergh, 2010) and secondary spinal motor neurons (Kawakita A, Sato K, Makino H, Ikegami H, & Takayama S, 2008; Svoboda, Vijayaraghavan, & Tanguay, 2002). Other nicotine-associated complications include respiratory infections, otitis media, asthma, childhood cancer, hearing loss, dental caries, metabolic syndromes, conduct disorder, attention-deficit/hyperactivity disorder, poor academic achievement, and cognitive impairment, tachycardia and fetal hypoxia, fatal cardiac arrhythmias (R. Chen, Clifford, Lang, & Anstey, 2013; Cook & Strachan, 1997; DiFranza, Aligne, & Weitzman, 2004; Ey et al., 1995; Haberg, Stigum, Nystad, & Nafstad, 2007; Liebrechts-Akkerman et al., 2011; Owen et al., 1993; Strachan & Cook, 1998; Zhou et al., 2014). The vulnerability to nicotine changes with age, but the mechanisms and the developmental origin of these health problems remain unknown and therefore late-onset diseases are not preventable. As mentioned earlier, nicotine is an agonist for acetylcholine receptors. The latter are also present on surfaces on non-neuronal cells and play important roles in cellular homeostasis. Nicotine binding to those receptors on non-neuronal cells can lead to cell division, arrest, or apoptosis in a context dependent manner (Table 1.2) (Zeidler, Albermann, & Lang, 2007). This

interferes with the normal signaling and can result in pathologies such as cancers (Grando, 2014), neurodegeneration (Durazzo, Mattsson, & Weiner, 2014; Fredriksson & Archer, 2004; Hernan, Takkouche, Caamano-Isorna, & Gestal-Otero, 2002; James, 2013; Smith, Dwoskin, & Pauly, 2010; Wei, Alberts, & Li, 2014; Zhou et al., 2014), and apoptosis in cardiovascular (L. Wang et al., 2014), pulmonary (Demiralay, Gursan, & Erdem, 2006), and pancreatic tissue (Bruin, Gerstein, Morrison, & Holloway, 2008; Bruin, Kellenberger, Gerstein, Morrison, & Holloway, 2007). Exposure to nicotine is also linked to diabetes, obesity, reduced fertility, etc. (Bruin, Gerstein, & Holloway, 2010).

Nicotine, MAPK and Sirtuins

Mitogen activated protein kinases (MAPK) signaling pathway is one of the most ancient and conserved signaling among eukaryotes. It is considered one of the central hubs that have a role in many functions like the regulation of gene expression, metabolism, cell division, survival, differentiation, and cell death. There are four conventional MAPKs (i.e. ERK1/2, p38, JUN1/2/3, ERK5) that are comprised of a Ser/Thr kinase domain flanked by an N and C-terminal regions of variable lengths. Each of the four groups is composed of a three module kinase such that the signal is relayed from the extracellular environment to a GTP-binding protein which triggers a sequential activation starting by MAPK, to MAPKK, and then MAPKK (Figure 1.2). MAPK signaling is enriched in nicotine disorders as noted in KARG (Knowledgebase for Addiction Related Gene) (C. Y. Li, Mao, & Wei, 2008). In particular, the ERK module was the first to be cloned and is among the most studied pathway (Cargnello & Roux, 2011). Commonly abused drugs modify ERK1/2 in critical neuro-regions related to reinforcement (Zamora-Martinez & Edwards, 2014). Nicotine was consistently shown to activate ERK1/2 in lung cancer cells (Heusch & Maneckjee, 1998), mouse primary auditory cortex (Intskirveli & Metherate, 2012),

nasopharyngeal carcinoma cells (Shi et al., 2012), mouse primary cortical neurons (Steiner, Heath, & Picciotto, 2007), bovine adrenal chromaffin cells (Sugano et al., 2006), spinal cord neurons (Toborek et al., 2007), human bronchial epithelial cells (Tsai et al., 2006) and vascular smooth muscle cells (Di Luozzo et al., 2005). On the other hand, it was downregulated in nicotine-treated cardiomyoctes (L. Wang et al., 2014).

Sirtuins are NAD-dependent class III protein and histone deacetylases that are universally found from bacteria to humans (Colak et al., 2011). It mediates different stress responses and is involved in lifespan extension in some organisms (Howitz et al., 2003). They are highly involved in cellular and organismal energy metabolism and are therefore responsive to caloric restriction by insulin signaling-dependent and independent mechanisms (X. Li & Kazgan, 2011). In addition, sirtuins contribute to and regulate the epigenetics signature of the genome and impact chromatin remodeling which results in global changes in gene expression. They have also been shown to be implicated in cardiovascular disease, cancer, neuronal function and neurodegenerative diseases (Nakagawa & Guarente, 2011) (Figure1.3). SIRT1 (silent information regulator) is one of the seven sirtuins and is the best and most studied (Colak et al., 2011). Nicotine reduced SIRT1 expression in A/J mice lungs (Iskandar et al., 2013), mouse neural stem cells (H. Lee et al., 2014), and cultured mouse embryos (Lin et al., 2012). Cigarette smoking also downregulated SIRT1in the mice lungs (Hwang, Sundar, Yao, Sellix, & Rahman, 2014) and in (Hwang, Yao, Caito, Sundar, & Rahman, 2013), lung epithelial cells, endothelial cells, and macrophages (Arunachalam, Yao, Sundar, Caito, & Rahman, 2010; Hwang et al., 2013; S. R. Yang et al., 2007). On the other hand, nicotine upregulated SIRT1 expression in human gingival fibroblasts (Park et al., 2013).Interestingly, SIRT1 was upregulated in normal bronchial

airway epithelial cells, but its downregulation was confined to cancerous lung tissue from smokers (Beane et al., 2012).

Nicotine and miRNAs

Consistent with the hypothesis of "developmental origins of adult health and disease" (DOHaD), early nicotine exposure increases the risks and occurrences of late-onset diseases. Epigenetics alterations have been shown to occur in children exposed to nicotine in utero (Breton et al., 2009) and were proposed to mediate consequential late-onset diseases. Epigenetic and non-genetic factors that influence intra and transgenerational inheritance include microRNAs. Though a very recent field, it significantly contributed to the epigenetic-based DOHaD data gap (Haugen, Schug, Collman, & Heindel, 2015). This is supported by evidence that showed changes of microRNA profiles in smokers' sperm samples (Marczylo, Amoako, Konje, Gant, & Marczylo, 2012). Moreover, our lab recently identified nicotine-responsive microRNAs that are consistently or specifically altered across three generations (F. A. Taki, Pan, Lee, & Zhang, 2014; F. A. Taki, X. Pan, & B. Zhang, 2013).We also performed a metaanalysis based on 118 studies concerned with microRNAs in drugs of abuse, including nicotine (F. A. Taki, Pan, & Zhang, 2015). We will discuss our analyses on nicotine in the following sections.

microRNAs: definition and biogenesis

Ever since their initial discovery in *C. elegans* in 1993 (R. C. Lee, Feinbaum, & Ambros, 1993), microRNAs have become universal molecules among eukaryotes. Due to their conservation and significant abundance (i.e. up to 50,000 copies/cell), miRNAs are considered to have significant biological roles. Indeed, with a deceptive size of 22 nucleotides, miRNAs fine tune the expression of a wide range of target genes involved in physiological and stress response

pathways (B. Zhang, Wang, & Pan, 2007). Initially, miRNAs were discovered as central hubs in development (e.g. let-7, lin-4) with strong phenotypic consequences. Soon thereafter, research uncovered their pleiotropic nature and contribution to a variety of conserved phenomena which include, but are not limited to, aging, cancer, metabolism, cell fate and differentiation, and neuro- and cardiovascular physiologies and disorders. Fourteen years later, Sathyan et al. uncovered a role of miRNAs as mediators of alcohol's teratogenic effects (Sathyan, Golden, & Miranda, 2007) and was followed with a burst in research on a variety of drugs of abuse and model organisms. Currently, miRNAs are known to protect from or promote substance use disorders (SUDs) and thus serve as attractive tools for therapeutic and/or diagnostic purposes.

Adopting miRNAs in the biomedical field necessitates understanding their biogenesis, regulation, and mode of action. Recently, a number of reviews have elegantly summarized the latest findings on miRNA-related mechanisms ((Fiore, Khudayberdiev, Saba, & Schratt, 2011; Ha & Kim, 2014; Leung & Sharp, 2010; Vidigal & Ventura, 2014; Wilczynska & Bushell, 2015). Briefly, miRNA-sequences are generally dispersed in coding (e.g. ORF) or non-coding regions (e.g. introns) (Ha & Kim, 2014). RNA pol II/III transcribe the DNA sequence to a capped and a potentially polyadenylated primary miRNA transcript (Pfeffer et al., 2005). This pri-miRNA transcript is about 1000 nucleotides in length and is comprised of one to several stem-loop structures, each of which is flanked by single stranded segments. Cleavage takes place at the intersection with flanking sites by an RNaseIII enzyme known as DROSHA (V. N. Kim, 2005). The liberated ~60-70 nt stem-loop molecule gives rise to a precursor miRNA (pre-miRNA) that is transported from the nucleus via Ran-GTP exportin5 complex. Further processing occurs in the cytoplasm by another RNaseIII enzyme known as Dicer which cleaves the pre-miRNA loop and liberates a partially matched miRNA:miRNA* duplex. The duplex is then actively unwound

to give rise to a guide strand which joins argonautes in a complex with TNRC6A–C (GW182) and other proteins to form miRISC (miRNA RNA interference complex). To find a specific target sequence, miRNAs act as guides that direct the interference machinery to MREs (microRNA response elements) residing in 3'-UTRs (B. Zhang et al., 2007), 5' UTR, or even ORF of a target mRNA (Stroynowska-Czerwinska, Fiszer, & Krzyzosiak, 2014). With such proximity, miRISCs were traditionally thought to silence genes through two general models: translational repression and/or mRNA decay. On the translational level, miRISC could compete with eIF4E and therefore prevent the recruitment of the translational machinery (Mathonnet et al., 2007). More so, miRISC could disrupt the sequential order of events at translational initiation by interacting with eIF6 (Chendrimada et al., 2007; Fabian & Sonenberg, 2012). On the other hand, destabilization of the mRNA could directly be due to miRNA-induced decapping and/or deadenylation (Behm-Ansmant et al., 2006). So, miRNA-dependent mRNA destabilization could be direct or indirect (translational repression) and has been shown to promote a dominant irreversible suppression (Bazzini, Lee, & Giraldez, 2012; Carthew & Sontheimer, 2009; Eichhorn et al., 2014). Meanwhile, miRISC could delay translation by holding a transcript in custody, after which it can be released to resume expression in response to certain cues (Kundu, Fabian, Sonenberg, Bhattacharyya, & Filipowicz, 2012). All in all, the maturation of a functional miRNA requires such canonical or other non-canonical pathways to fine-tune gene expression (F. A. Taki et al., 2015) (Figure 1.3).

miRNA nomenclature

Animal miRNA nomenclature includes the host species (i.e. first three letters), miR-, and a number. The latter is based on the order of discovery. However, interspecies miRNAs with identical sequences will usually get the same number. Unfortunately, several miRNAs sharing

high degree of homology (≥ 70%) are often not annotated with similar nomenclature. For example, cel-miR-58 family (cel-miR-58, cel-miR80, cel-miR-81, cel-miR-82, cel-miR-1018, cel-miR-1022) in *C. elegans* is homologous to dme-bantam and miR-306* in drosophila as well as mir-450b-3p in humans (Ibanez-Ventoso, Vora, & Driscoll, 2008). In addition, miRNA guide strands were distinguished from the passenger strand that was identified as miRNA* (e.g. miR_St, miR-as). This is based on the relative abundance of the miRNA strand. Unfortunately, due to some data gaps in abundance data, miRNA are sometimes referred to as miR-5p and miR-3p. This is problematic because the duplex strands are not completely identical and have different target sets (Ambros et al., 2003; Kozomara & Griffiths-Jones, 2014). Since studies usually refer to miRNAs by their names, rather than their stable accession number or sequence, it is a challenge to perform meta-analyses to identify commonly altered miRNAs across species in response to one or more drugs of abuse. Therefore, some biologically significant miRNAs might be missed due to differences in nomenclature.

Complexity of miRNA regulation and functional validation

Most miRNA mutants are phenotypically 'normal'. This has prompted not only skeptics, but also leaders in the field to declare that "most miRNAs are individually not essential for development or viability" (Alvarez-Saavedra & Horvitz, 2010; Miska et al., 2007). Nevertheless, recent research highlighted the complexity of miRNA-dependent regulation. miRNA profiles are specific to treatment, exposure duration, cell compartments, cell, tissue, organ types, developmental period, physiological state and history, environment, ethnicity, genetic polymorphism, species, etc… Thus, when assessing their functions, miRNAs should be studied as a system in a dynamic transcriptome. If we consider miRNAs as one non-linear system, then a small or large change in a miRNA(s) will not necessarily result in a proportional effect on the

system as a whole (Rickles, Hawe, & Shiell, 2007). miRNAs are "epitranscriptional regulators" (G. W. Dorn, 2013; Y. Hu et al., 2012) interacting directly with only a subset of master regulators of gene expression (e.g. transcription factors (Matkovich, 2014)) which amplify the initial modest signal. Thus, initial conditions shape a miRNA mosaic which in turn works as a system to canalize sensitive biological events (e.g. cell cycle, differentiation) (G. W. Dorn, 2nd, 2012). So, one setting encompasses not a single, but a repertoire of miRNAs which contribute to large scale and multi-level impacts (e.g. transcript, protein levels).

The vagueness of miRNA behavior stems from: i) dynamic regulation of miRNA biogenesis, ii) versatility of miRISC gene regulation, iii) synergism vs. antagonism with other RNA (e.g. circRNA, lncRNA) and RNP (e.g. HuR), iv) ambiguity in miRNA-target binding sites (e.g. 3'/5' UTR, ORF), v) context-dependent modes of target regulation (e.g. mRNA decay or translational repression), vi) shifts between activation and repression of target expression, vii) target diversity for a single miRNA, viii) miRNA redundancy for a single target (Breving & Esquela-Kerscher, 2010), and ix) reversed regulatory outcomes as a function of miRNA competition (Nyayanit & Gadgil, 2015). With this in mind, it is a challenge to dissect miRNA pathways or to deduce causal relationships. In fact, as the complexity of a system increases, our ability to precisely predict its behavior decreases (Ripoli, Rainaldi, Rizzo, Mercatanti, & Pitto, 2010; Zadeh, 1973). This concept can be applied to miRNAs in conferring robustness in noisy, crowded, and chaotic living systems in response to fluctuating signals (Ripoli et al., 2010). Interestingly, we found support for this idea in our meta-analysis. Morphine induces miR-23b through MOR, which in turn is targeted by miR-23b through a negative loop while its antagonist also upregulated miR-23b in cells in a MOR-dependent and - independent manner (Q. Wu, Zhang, Law, Wei, & Loh, 2009). As in other studies, cocaine-responsive miRNAs had opposite

directional changes, yet shared common target. Therefore, the directions of miRNA targets could not be anticipated (Eipper-Mains et al., 2011). In addition, many candidate targets will be missed when considering only inversely expressed miRNAs. In addition, choosing miRNAs with highest differential changes is not necessarily the best route as critical drug-induced miRNAs might undergo modest shifts in expression levels due to their abundant nature. Sometimes two opposing miRNAs like miR-21 (anti-apoptotic) and miR-335 (pro-apoptotic) have the same directional change in response to a drug like alcohol, yet the net effect was proliferation (Sathyan et al., 2007). Noteworthy, some targets are commonly regulated by different miRNAs with opposite expression levels. So, direction of change of potential targets can't be anticipated (Eipper-Mains et al., 2011).

The miRNA response to drugs of abuse has been well elucidated across many biological systems ((Chandrasekar & Dreyer, 2009, 2011; Hollander et al., 2010; Jadhav et al., 2013; Pietrzykowski et al., 2008; Rajasethupathy et al., 2009; Saba et al., 2012; Schaefer et al., 2010). Most of the studies focused on alcohol (41%) followed by tobacco/nicotine (20%), stimulants (17%), opioids (13%), anesthetics (6%), and lastly cannabinoids (3%). Tobacco/nicotine was the only group that had a higher percentage of studies reporting global downregulation (~50%) of miRNAs (Figure 1.5A). Nevertheless, global upregulation was still reported in about 33% of the tobacco/nicotine studies while 17% reported equal distribution of miRNA expression levels (Figure 1.5B). Interestingly, tobacco/nicotine affected the highest percentage of miRNAs (13%) relative to the other drugs of abuse (Figure 1.6). Only 2 out of 205 miRNAs were consistently upregulated in response to nicotine. These were let-7 family, and miR-21 (Table 1.3). In addition, let-7 was also repeatedly reported to be downregulated by nicotine, while miR-125b and miR-34b were the only consistently downregulated miRNAs (Table 1.4).

A comprehensive list of miRNA-dependent mechanisms are summarized in (Table 1.5). For example, nicotine affected miRNAs involved in stem cell regeneration and cellular proliferation in human periodontal ligament-derived stem cells (Ng et al., 2013), human gastric adenocarcinoma (Shin et al., 2011) and bronchial epithelial cells (Kassie, Jarcho, & Endalew, 2010). It also antagonized the effects of alcohol on select miRNAs in fetal mouse cerebral cortical neural precursors (Balaraman, Winzer-Serhan, & Miranda, 2012b) and affected miRNA mediators of neuroplasticity in rat adrenal pheochromocytoma (W. Huang & Li, 2009). In higher organisms, nicotine affected miRNAs involved in atrial remodeling in canines (Shan et al., 2009). miR-21 served a protective role in mice and human tissue for AAA (Abdominal Aortic Aneurysm) disease in response to nicotine (Maegdefessel et al., 2012). Recently, we showed that post-embryonic nicotine exposure altered global miRNA expression profiles upon direct nicotine exposure (F. A. Taki et al., 2013) and this effect was transgenerational (F. A. Taki et al., 2014).

Are they a cause or a side-effect (symptom) of SUD (Substance Use Disorder)?

Substance use disorder is a broad term that describes a variety of effects. Determining whether miRNA dysfunction causes or results from SUD has been recognized as a conundrum (Fiore et al., 2011). It is best to tackle this question relative to a phenotype of interest. Even then, it is difficult to determine sequence of events, as miRNAs sometimes act as a double-edged sword. For example, HIV infection can alter miRNAs like miR-9 which can make CNS neurons susceptible to methamphetamine addiction (Tatro et al., 2013). On the other hand, cocaine downregulates anti-HIV miRNAs like miR-155 and miR-125b, thereby enhancing HIV replication (Mantri, Pandhare Dash, Mantri, & Dash, 2012; Napuri et al., 2013). This is mainly because they are involved in auto-regulatory loops. For example, morphine activates MOR which activates a signaling pathway resulting in the upregulation of miR-339-3p (Q. Wu et al.,

2013) and miR-23b (Q. Wu et al., 2009), both of which target MOR itself at its 3' UTR. Meanwhile, not all DA-responsive miRNAs are pathogenic, but instead some have a protective role. For example, nicotine potentiated the increase of miR-21 in AAA (Abdominal Aortic Aneurysm) mice models. miR-21 is usually described as an oncomir (Medina, Nolde, & Slack, 2010), yet in the context of AAA disease, it protected from aortic expansion due to increased smooth muscle cell proliferation. When AAA mice received both nicotine and anti-miR-21, they died due to the rupture of enlarged aortic wall (Maegdefessel et al., 2012).

Meanwhile, the regulation of miRNA levels is not limited to transcription, but can also happen at any of the steps during biogenesis (e.g. DROSHA, DGCR8, XPO5, RAN, DICER1, TARBP2, AGO1, AGO2, GEMIN3, GEMIN4 (Gedik et al., 2014)). Drugs can affect Drosha/Pacha microprocessor or dicer enzyme, which subsequently alters the production of precursor miRNA (pre-miRNA) or mature miRNAs, respectively. Some drugs have been shown to affect argonautes which consequently alter the activity of miRISC complex. For example, Gedik et al. reported AGO1 rs595961, AGO2 rs4961280 G alleles and GEMIN4 rs910924 T-alleles to affect the susceptibility to alcohol dependence (AD) in a Turkish population (Gedik et al., 2014). In addition, an increase in Drosha, Dicer and Ago2 was observed in zebrafish treated with cocaine (Lopez-Bellido, Barreto-Valer, Sanchez-Simon, & Rodriguez, 2012). Alcohol also increased Drosha in the dorsal hippocampus of rats, while it increased Drosha and Dicer in the dorsal and ventral hippocampus (Prins, Przybycien-Szymanska, Rao, & Pak, 2014). These changes might result in non-specific miRNA alterations and thus it is easier to consider them as side-effects of drug exposure, assuming that select miRNAs themselves are not direct co-targets. Noteworthy, current technical advancements and tools do allow for the diagnosis of miRNA dysfunction from transcription, through biogenesis, until its loading to RISC. For example

antisense oligonucleotides can be used to manipulate pri-miRNAs, pre-miRNAs, or mature miRNAs. Complete or conditional knock outs can be established for any gene of interest involved in the biogenesis pathway (e.g. Drosha, Dicer) (McNeill & Van Vactor, 2012). Taken collectively, we refer to the description by Li, & van der Vaart which considered miRNAs as middlemen for addiction 'tug of war' between motivation-based learning versus counteractive neuronal homeostasis (M. D. Li & van der Vaart, 2011).

microRNA transport

Recently, miRNAs have been reported to have extra/inter-cellular and even between individuals (e.g. placenta, lactation) shuttling through vesicles including exosomes, apoptotic bodies, RNA binding proteins, and lipoproteins (Boon & Vickers, 2013; H. J. Lee, 2014; Rayner & Hennessy, 2013). Not all extracellular miRNAs are byproducts of cell damage or rupture. Others are nevertheless absent in the parent cell as they are destined to be secreted after transcription. For example, miR-122 was downregulated in liver tissue, but upregulated in exosome fractions as well as in the serum of alcohol liver disease models (Bala et al., 2012). Interestingly, treating SIV-infected SH-SY5Y neuronal cells with morphine did not affect PDGF-B chain levels. However, Hu et al. elegantly showed that morphine potentiated SIV-mediated neuronal apoptosis by downregulating tropic factor PDGF-B chain in neurons in a cell non-autonomous manner. This required exosome-dependent shuttling of miR-29b from astrocytes to exert its action on PDGF-B in the neurons (G. Hu et al., 2012). Thus, miRNAs can also serve as signaling molecules involved in orchestrating organism level communications and processes. This raises an issue with analyses that try to exclude predicted miRNA targets that are not co-expressed within tissue of interest. Meanwhile, it adds another challenge to target prediction approaches. Similarly, whole cell miRNA profiling fails to differentiate between

nuclear and cytoplasmic miRNA fractions. Indeed, miRNAs have also been shown to have a nuclear presence (Roberts, 2014). Thus, those miRNAs might be shuttled back and forth between the cytoplasm and the nucleus to silence nascent transcripts (Benhamed, Herbig, Ye, Dejean, & Bischof, 2012; Liang, Zhang, Zen, Zhang, & Chen, 2013), to mediate epigenetic remodeling (D. H. Kim, Saetrom, Snove, & Rossi, 2008) at promoters of gene and/or other non-coding RNAs, and to perform other roles that are not yet identified. Current research has not yet accounted for predicting and validating miRNAs as transcriptional regulators of other miRNAs (Liang et al., 2013; Tang et al., 2012). Collectively, miRNAs can serve as diagnostic markers (e.g. salivary miRNAs for oral diseases (H. J. Lee, 2014)) and therapeutic agents (Dreyer, 2010; Eipper-Mains, Eipper, & Mains, 2012; Jadhav et al., 2013; Pietrzykowski, 2010) for nicotine-induced diseases.

Caenorhabditis elegans

C. elegans (Phylum Nematoda: Family Rhabditidae) is a free-living nematode that was first isolated by Maupus in 1990 (Maupus, 1990). Soon thereafter, it was established as a genetics model organism by Sydney Brenner (S. Brenner, 1974). It is our preferred model due its short generation time of about 2-3 days at 20 °C. Eggs hatch to L1 larvae of about 250 µm and go through four larval stages before molting to adulthood (1mm) (Z.F. Altun & D.H. Hall, 2009) (Table 1.6). Spermatogenesis occurs in late L4 stage (L'Hernault, 2006) and is followed by oogenesis in young adult hermaphrodites. Thus, we considered the L1 to L3/L4 molt period to model post-embryonic development (i.e. lacking differentiated gametes). The self-fertilizing hermaphrodites lay around 300 eggs which can increase to around 1000 eggs after mating. With this in mind, the occurrence of males in an N2 population is about 0.1% (Ward & Carrel, 1979). This increases in response to stressful conditions like starvation, crowding, shifts in temperatures. While males are important to introduce allele diversity as a survival strategy in the wild, they are

used as tools to introduce alleles of interest in cross linking experiments (McGraw-Hill-Higher-Education, 2011).

Despite being a simple multicellular organism, the worm enables studying complex behavior and syndromes. It has been used in many recent phenology studies (McGary et al., 2010; van der Velde et al., 2014) to understand human maladies like cancer (Cha, Datla, Hollis, Kimble, & Lee, 2012; Kirienko, Mani, & Fay, 2010; Kobet et al., 2014), ageing (Honda, Honda, Narici, & Szewczyk, 2014), development (Hubbard, Korta, & Dalfo, 2013; Pilon, 2014; Schaedel, Gerisch, Antebi, & Sternberg, 2012), addiction (Feng et al., 2006; Sellings et al., 2013; F. Taki, X. Pan, & B. Zhang, 2013; F. A. Taki et al., 2014; F. A. Taki et al., 2013; Zhu, Zhang, & Li, 2014) and other cardiovascular (Diomede et al., 2014; Epstein & Benian, 2012) and neurodegenerative diseases like Alzheimer's (Ewald & Li, 2009) and Huntington (Voisine et al., 2007). As the first multicellular organism to have its nervous system delineated, the worm with its 302 neurons offers an attractive platform to understand universal phenomenon like sleep (Cho & Sternberg, 2014; A. J. Hill, Mansfield, Lopez, Raizen, & Van Buskirk, 2014) and pain (Riera et al., 2014) research. Each neuron can be labelled by a specific marker which facilitates identification and tracking. More so, neuron ablations are non-lethal in the worm under standard laboratory conditions (Riddle, Blumenthal, & B.J., 1997), a characteristic that is challenging and/or ethically controversial in higher organisms.

Research on the worm has received much attention in the biomedical field especially after two Nobel Prize awards in medicine and physiology in 2002 and 2006. This is due to the conservation of major signaling pathways as well as high genome homology (~80%) with humans (Carroll, Dougherty, Ross-Macdonald, Browman, & FitzGerald, 2003; Consortium, 1998; Shaye & Greenwald, 2011). In our research, we focus on miRNA profiling and functional

annotation. With this in mind, we took advantage of *C. elegans* having only 1/6th of the total number of miRNAs relative to humans (Kozomara & Griffiths-Jones, 2014). Such a feature enables us to perform systematic profiling using gold-standard single-plex qRT-PCR. Another advantage of using *C. elegans* in drugs of abuse research is that we can control for the effects of conditioning. In other words, drug addiction is also impacted by social and habitual cues that enhance the urge to abuse. Thus, it becomes challenging to dissociate those cues from the direct physiological and pharmacological effects of the drug (N. L. Benowitz, 2009). So, treating the worms with nicotine will allow us to focus on the molecular basis of the drug-induced effects.

Drug absorption in the worm

1. Under normal conditions, the average time for residence of a bacterium in the worm intestine is less than two minutes (McGhee, 2007).
2. The pH levels estimated in *C. elegans* intestine is about 3.59 (Chauhan, Orsi, Brown, Pritchard, & Aylott, 2013) in comparison to the human blood and intestines of which pH ranges from 6 to 7.4 (Fallingborg, 1999). This impacts the efficiency of nicotine absorption since it is a weak base that is ionized in acidic environments (i.e. worm's intestine) and is assimilated at a much slower rate (N. L. Benowitz, 2009).
3. Previous studies that exposed worms to about 2% alcohol showed that the inner concentration was comparable to 0.1% human blood alcohol levels (Davis, Li, & Rankin, 2008). It is noteworthy to mention that alcohol readily permeates the cuticle in *C. elegans* (Mitchell et al., 2007) and in spite of this property, a 20 fold decrease was still observed between external and internal environments. Meanwhile, the cuticle is a barrier for nicotine (Ruiz-Lancheros, Viau, Walter, Francis, & Geary, 2011) and can thus decrease the internal concentration even further.

4. The bioavailability of the drug (i.e. nicotine) is affected by the method of exposure. The worm's metabolic rate is proportional to the drug dose and reaches its peak after 8-12 hours of exposure. Also, the concentration of the drug decreases with time and is impacted by whether the food supplied is killed versus living bacteria. In addition, drugs infused to solid NGM media which is seeded with living bacteria resulted in the second lowest absorption rate in the worm (Zheng, Ding, Li, Wu, & Luo, 2013). The latter was our method of exposure and therefore together with the ionized state of nicotine in the worm's acidic intestine and rapid digestive and metabolic rates of the worm, the inner nicotine concentration is expected to more than 20 folds lower than the nicotine concentration in the medium.

Pharynx and heart

C. elegans is a filter feeder which takes up food from the environment to the pharynx before being transported to the intestines. The pharynx is not directly exposed to the environment because its lumen is lined with the cuticle that connects to the epidermis. The pharynx is divided into three main parts: corpus, isthmus, and terminal bulb. Food is passed along through two actions known as pumping and peristalsis. The former depends on the consecutive contraction and relaxation of the radial muscles, respectively. This is followed by an intermittent peristalsis that moves partially broken food from the anterior isthmus to the terminal bulb. Food is then subjected to further crushing by the grinder in the terminal bulb before being passed to the intestines. Noteworthy, the grinder movement is proportional to terminal bulb contraction and relaxation and is used as a proxy to count pumping rate in our pumping assay. A single "pump" corresponds to an action potential that depends on five ion channels: nAChR, low and high-threshold voltage-gated calcium channels, glutamate-gated chloride channel, and voltage-gated

potassium channel (Avery & You, 2012; Mango, 2007). So, despite having different purposes, processing the different signals for a synchronized action is physiologically similar to the human heart as will be discussed below.

Though the pharynx and the heart are not derived from the same germ line origin (ectoderm vs mesoderm, respectively), they are considered to be similar by convergent evolution (Mango, 2007). Both are tube-like structures, with a lumen lined with binucleated muscle cells. More so, both undergo autonomous rhythmic pumping which can occur even without nervous input. However, neurons are required for efficient functioning (e.g. feeding) (Avery & You, 2012). The pharynx serves as a simple platform to understand how environmental stimuli are received by neuro-signaling pathways which in turn relay the signal to motor function. Thus the frequencies of both pumping and peristalsis, described above, are outputs of endogenous and exogenous signals that trigger neuro-muscular interactions (Song & Avery, 2013).

Germline apoptosis

Development is a balance between programmed cell growth and death (i.e. apoptosis). The latter is important to maintain homeostasis, and eliminate aberrant, damaged or harmful cells. The dysregulation of apoptosis is associated with many human diseases (Kam & Ferch, 2000). For example, inhibition of apoptosis is a hallmark of cancer, while excessive apoptosis is associated with heart and some neurodegenerative diseases like Alzheimer's as well as diabetes if it occurred in pancreatic beta-islet cells. Apoptosis was first discovered in *C. elegans* which resulted in the first Nobel Prize. The worm undergoes two types of apoptosis: physiological and stress induced. The former progresses independently from environmental cues and follows a fixed and punctual program in select somatic (e.g. NSM sister cells) and germ cells. On the other

hand, apoptosis in other germ cells is not encoded, but instead reflects the worm's life history and experiences (Gartner A., Boag P. R., & B., 2008). Hence stress-induced germ cell apoptosis represents a highly tractable system to study apoptosis for prognostic, diagnostics, as well we therapeutic purposes. Below, I will briefly provide an overview of germ cell development and physiology that are relevant to our experimental design as well as apoptosis assay.

Germ cells differentiate around the four-cell stage during embryogenesis. However, further differentiation of the somatic gonad is coupled with that of germ cells and occurs post-embryonically. Those germ cell precursors require signaling and nutrients from the forming somatic gonad for subsequent steps. The process starts in mid-L1 until L3 stage where a somatic "niche" is formed from 12 cells: 2 distal tip cells (DTCs) and 10 proximal cells. This represents the backbone for subsequent bursts in germ and somatic cell proliferation and marks the beginning of sex determination. As worms grow to L4 and young adult stage, events continue to be spatiotemporal starting from DTC-dependent germ cells mitosis which then transition to meiosis around gonad loop region. This is the site of apoptosis and is known as the death loop. Until this point, germ cells are syncytial and share a cytoplasm during the pachytene stage. Thus, it is hypothesized that apoptosis would not only eliminate faulted germ cells, but they would also provide nutrients to the qualified cells through the shared cytoplasm (Hubbard & Greenstein, 2005). Interestingly, MAPK pathway represents a common factor that controls both the progression beyond pachytene as well as the apoptosis rate. Thus, decreased MAPK signaling is proportional to pachytene arrest, decrease in apoptosis, as well as a decrease in fertility. Hence, it is important to note that apoptosis does not always correlate with fertility, and in fact has been reported to be inversely proportion in some situations (Gartner A. et al., 2008).

How to differentiate the developmental (normal and physiological) apoptosis from the stress-induced apoptosis? Both types of apoptotic cells are restricted to pachytene cells (i.e. death loop region) in *C. elegans* and are morphologically indistinguishable. However, they are triggered by different molecular pathways. Stressors can be further categorized into either genotoxic or non-genotoxic, both of which have different upstream apoptotic signal transducers. Signaling vie cep-1 (i.e. P53 tumor suppressor) and egl-1 (BH3-only protein) is required for genotoxic apoptosis, but is not required for non-mutagenic agents/events. While physiological apoptosis represents the baseline, an increase in apoptotic count can be linked to worm exposure in comparison to those control worms at similar developmental stages (e.g. young vs. old) (Gartner A. et al., 2008).

Hypotheses and objectives

Previously, our lab performed a systematic and detailed behavioral analysis in *C. elegans* larvae. Nicotine treatment was limited to the early larval stages in the parental worms. However, I have found that those behavioral changes were not restricted to the parents, but also extended to offspring and grand-offspring generations (F. Taki et al., 2013). Based on these preliminary results, ***I hypothesized that (i) post-embryonic nicotine exposure impacts microRNA profiles across generations and that (ii) some microRNAs mediate nicotine-induced early-onset and late-onset diseases.*** To test the hypotheses, I performed the following aims. First, I identified a transgenerational effects on specific microRNA pathways. Then, I examined the molecular mechanisms for the intragenerational inheritance of the damages induced by nicotine.

- Aim I: To identify nicotine-responsive microRNAs that are consistently altered in more than one generation (Chapter II).

    - Objective 1: To perform global microRNA profiling for parent (F0), offspring (F1), and grand-offspring (F2) worm generations using single-plex qRTPCR.
    - Objective 2: To identify miRNA-enriched biological networks in response to early nicotine exposure based on microRNA-target enrichment and bioinformatics functional annotation. This sets the basis for investigating the MS80 (mpk-1, sir-2.1, and miR-80) pathway.
- Aim II: To examine the roles of MS80 (mpk-1, sir-2.1, miR-80) in nicotine-induced early-onset phenotypes (Chapter III).
    - Objective 1: To investigate the role of MS80 in nicotine-induced developmental arrest. This was based on reverse genetics, treatment with antagonists, imageJ software and microscopic analyses.
    - Objective 2: To investigate the role of MS80 in nicotine-induced pharyngeal pumping inhibition. This was based on reverse genetics, treatment with inhibitors, and behavioral analysis.
- Aim III: To validate the role of MS80 (mpk-1, sir-2.1, miR-80) in nicotine-induced late-onset diseases (Chapter IV).
    - Objective 1: To examine the effects of post-embryonic nicotine exposure on adult reproduction, lifespan, and germ line apoptosis. This involved reproduction and lifespan assays, and staining and counting apoptotic cells.
    - Objective 2: Investigate the role of MS80 in nicotine-induced adult germ line apoptosis. This was based on reverse genetics, treatment with inhibitors, SYTO12 and acridine orange staining and microscopic analyses.

- Aim IV: To deduce a possible functional relationship among mpk-1, sir-2.1, and miR-80 in response to early nicotine exposure (Chapter V).

References

Altun, Z. F., & Hall, D. H. (2009). Introduction. In WormAtlas. doi:10.3908/wormatlas.1.1, from http://www.wormatlas.org

Alvarez-Saavedra, E., & Horvitz, H. R. (2010). Many families of *C. elegans* microRNAs are not essential for development or viability. [Research Support, Non-U.S. Gov't]. *Curr Biol, 20*(4), 367-373. doi: 10.1016/j.cub.2009.12.051

Ambros, V., Bartel, B., Bartel, D. P., Burge, C. B., Carrington, J. C., Chen, X., . . . Tuschl, T. (2003). A uniform system for microRNA annotation. *RNA, 9*(3), 277-279.

Arunachalam, G., Yao, H., Sundar, I. K., Caito, S., & Rahman, I. (2010). SIRT1 regulates oxidant- and cigarette smoke-induced eNOS acetylation in endothelial cells: Role of resveratrol. [Research Support, N.I.H., Extramural]. *Biochem Biophys Res Commun, 393*(1), 66-72. doi: 10.1016/j.bbrc.2010.01.080

Avery, L., & You, Y. J. (2012). *C. elegans* feeding. *WormBook*, 1-23. doi: 10.1895/wormbook.1.150.1

Bala, S., Petrasek, J., Mundkur, S., Catalano, D., Levin, I., Ward, J., . . . Szabo, G. (2012). Circulating microRNAs in exosomes indicate hepatocyte injury and inflammation in alcoholic, drug-induced, and inflammatory liver diseases. [Research Support, N.I.H., Extramural]. *Hepatology, 56*(5), 1946-1957. doi: 10.1002/hep.25873

Balaraman, S., Winzer-Serhan, U. H., & Miranda, R. C. (2012). Opposing actions of ethanol and nicotine on microRNAs are mediated by nicotinic acetylcholine receptors in fetal cerebral cortical-derived neural progenitor cells. *Alcohol Clin Exp Res, 36*(10), 1669-1677. doi: 10.1111/j.1530-0277.2012.01793.x

Bazzini, A. A., Lee, M. T., & Giraldez, A. J. (2012). Ribosome profiling shows that miR-430 reduces translation before causing mRNA decay in zebrafish. *Science, 336*(6078), 233-237. doi: 10.1126/science.1215704

Beane, J., Cheng, L., Soldi, R., Zhang, X., Liu, G., Anderlind, C., . . . Bild, A. H. (2012). SIRT1 pathway dysregulation in the smoke-exposed airway epithelium and lung tumor tissue. [Research Support, N.I.H., Extramural]. *Cancer Res, 72*(22), 5702-5711. doi: 10.1158/0008-5472.CAN-12-1043

Behm-Ansmant, I., Rehwinkel, J., Doerks, T., Stark, A., Bork, P., & Izaurralde, E. (2006). mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes. *Genes Dev, 20*(14), 1885-1898. doi: 10.1101/gad.1424106

Benhamed, M., Herbig, U., Ye, T., Dejean, A., & Bischof, O. (2012). Senescence is an endogenous trigger for microRNA-directed transcriptional gene silencing in human cells. [Research Support, Non-U.S. Gov't]. *Nat Cell Biol, 14*(3), 266-275. doi: 10.1038/ncb2443

Benowitz, N. L. (2009). Pharmacology of nicotine: addiction, smoking-induced disease, and therapeutics. *Annu Rev Pharmacol Toxicol, 49*, 57-71. doi: 10.1146/annurev.pharmtox.48.113006.094742

Benowitz, N. L., Hukkanen, J., & Jacob, P., 3rd. (2009). Nicotine chemistry, metabolism, kinetics and biomarkers. *Handb Exp Pharmacol*(192), 29-60. doi: 10.1007/978-3-540-69248-5_2

Boon, R. A., & Vickers, K. C. (2013). Intercellular transport of microRNAs. *Arterioscler Thromb Vasc Biol, 33*(2), 186-192. doi: 10.1161/ATVBAHA.112.300139
Brenner, S. (1974). The genetics of Caenorhabditis elegans. *Genetics, 77*(1), 71-94.
Breton, C. V., Byun, H. M., Wenten, M., Pan, F., Yang, A., & Gilliland, F. D. (2009). Prenatal tobacco smoke exposure affects global and gene-specific DNA methylation. *Am J Respir Crit Care Med, 180*(5), 462-467. doi: 10.1164/rccm.200901-0135OC
Breving, K., & Esquela-Kerscher, A. (2010). The complexities of microRNA regulation: mirandering around the rules. *Int J Biochem Cell Biol, 42*(8), 1316-1329. doi: 10.1016/j.biocel.2009.09.016
Bruin, J. E., Gerstein, H. C., & Holloway, A. C. (2010). Long-term consequences of fetal and neonatal nicotine exposure: a critical review. *Toxicol Sci, 116*(2), 364-374. doi: 10.1093/toxsci/kfq103
Bruin, J. E., Gerstein, H. C., Morrison, K. M., & Holloway, A. C. (2008). Increased pancreatic beta-cell apoptosis following fetal and neonatal exposure to nicotine is mediated via the mitochondria. [Research Support, Non-U.S. Gov't]. *Toxicol Sci, 103*(2), 362-370. doi: 10.1093/toxsci/kfn012
Bruin, J. E., Kellenberger, L. D., Gerstein, H. C., Morrison, K. M., & Holloway, A. C. (2007). Fetal and neonatal nicotine exposure and postnatal glucose homeostasis: identifying critical windows of exposure. *J Endocrinol, 194*(1), 171-178. doi: 10.1677/JOE-07-0050
Cameron, P. (1967). The presence of pets and smoking as correlates of perceived disease. *J Allergy, 40*(1), 12-15.
Cargnello, M., & Roux, P. P. (2011). Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. *Microbiol Mol Biol Rev, 75*(1), 50-83. doi: 10.1128/MMBR.00031-10
Carroll, P. M., Dougherty, B., Ross-Macdonald, P., Browman, K., & FitzGerald, K. (2003). Model systems in drug discovery: chemical genetics meets genomics. [Review]. *Pharmacol Ther, 99*(2), 183-220.
Carthew, R. W., & Sontheimer, E. J. (2009). Origins and Mechanisms of miRNAs and siRNAs. *Cell, 136*(4), 642-655. doi: 10.1016/j.cell.2009.01.035
CDC. (1988). The Health Consequences of Smoking - Nicotine Addiction: A Report of the Surgeon General. Center for Health Promotion and Education. Office on Smoking and Health. (DHHS Publication No. (CDC) 88-8406). Retrieved from http://profiles.nlm.nih.gov/NN/B/B/Z/D/.
CDC. (2010). How Tobacco Smoke Causes Disease: The Biology and Behavioral Basis for Smoking-Attributable Disease: A Report of the Surgeon General. Atlanta (GA) Retrieved from http://www.ncbi.nlm.nih.gov/books/NBK53018/
Cha, D. S., Datla, U. S., Hollis, S. E., Kimble, J., & Lee, M. H. (2012). The Ras-ERK MAPK regulatory network controls dedifferentiation in Caenorhabditis elegans germline. [Research Support, Non-U.S. Gov't]. *Biochim Biophys Acta, 1823*(10), 1847-1855. doi: 10.1016/j.bbamcr.2012.07.006
Chandrasekar, V., & Dreyer, J. L. (2009). microRNAs miR-124, let-7d and miR-181a regulate cocaine-induced plasticity. *Mol Cell Neurosci, 42*(4), 350-362. doi: 10.1016/j.mcn.2009.08.009
Chandrasekar, V., & Dreyer, J. L. (2011). Regulation of MiR-124, Let-7d, and MiR-181a in the accumbens affects the expression, extinction, and reinstatement of cocaine-induced

conditioned place preference. [Research Support, Non-U.S. Gov't]. *Neuropsychopharmacology, 36*(6), 1149-1164. doi: 10.1038/npp.2010.250

Chauhan, V. M., Orsi, G., Brown, A., Pritchard, D. I., & Aylott, J. W. (2013). Mapping the pharyngeal and intestinal pH of Caenorhabditis elegans and real-time luminal pH oscillations using extended dynamic range pH-sensitive nanosensors. [Research Support, Non-U.S. Gov't]. *ACS Nano, 7*(6), 5577-5587. doi: 10.1021/nn401856u

Chekulaeva, M., & Filipowicz, W. (2009). Mechanisms of miRNA-mediated post-transcriptional regulation in animal cells. *Curr Opin Cell Biol, 21*(3), 452-460. doi: 10.1016/j.ceb.2009.04.009

Chen, R., Clifford, A., Lang, L., & Anstey, K. J. (2013). Is exposure to secondhand smoke associated with cognitive parameters of children and adolescents?--a systematic literature review. *Ann Epidemiol, 23*(10), 652-661. doi: 10.1016/j.annepidem.2013.07.001

Chendrimada, T. P., Finn, K. J., Ji, X., Baillat, D., Gregory, R. I., Liebhaber, S. A., . . . Shiekhattar, R. (2007). MicroRNA silencing through RISC recruitment of eIF6. *Nature, 447*(7146), 823-828. doi: 10.1038/nature05841

Cho, J. Y., & Sternberg, P. W. (2014). Multilevel modulation of a sensory motor circuit during *C. elegans* sleep and arousal. *Cell, 156*(1-2), 249-260. doi: 10.1016/j.cell.2013.11.036

Colak, Y., Ozturk, O., Senates, E., Tuncer, I., Yorulmaz, E., Adali, G., . . . Enc, F. Y. (2011). SIRT1 as a potential therapeutic target for treatment of nonalcoholic fatty liver disease. *Med Sci Monit, 17*(5), HY5-9.

Consortium, T. C. e. S. (1998). Genome Sequence of the Nematode *C. elegans*: A Platform for Investigating Biology. *Science, 282*(5396), 2012-2018. doi: 10.2307/2897605

Cook, D. G., & Strachan, D. P. (1997). Health effects of passive smoking. 3. Parental smoking and prevalence of respiratory symptoms and asthma in school age children. [Review]. *Thorax, 52*(12), 1081-1094.

Corbo, G. M., Agabiti, N., Forastiere, F., Dell'Orco, V., Pistelli, R., Kriebel, D., . . . Perucci, C. A. (1996). Lung function in children and adolescents with occasional exposure to environmental tobacco smoke. *Am J Respir Crit Care Med, 154*(3), 695-700. doi: 10.1164/ajrccm.154.3.8810607

Cotton, L. (1998). Tobacco: The Early History of a New World Crop, Accessed on March 8 2016 from: http://www.nps.gov/jame/learn/historyculture/tobacco-the-early-history-of-a-new-world-crop.htm

Davis, J. R., Li, Y., & Rankin, C. H. (2008). Effects of developmental exposure to ethanol on Caenorhabditis elegans. [Research Support, Non-U.S. Gov't]. *Alcohol Clin Exp Res, 32*(5), 853-867. doi: 10.1111/j.1530-0277.2008.00639.x

Demiralay, R., Gursan, N., & Erdem, H. (2006). The effects of erdosteine, N-acetylcysteine, and vitamin E on nicotine-induced apoptosis of pulmonary cells. *Toxicology, 219*(1-3), 197-207. doi: 10.1016/j.tox.2005.11.020

Di Luozzo, G., Pradhan, S., Dhadwal, A. K., Chen, A., Ueno, H., & Sumpio, B. E. (2005). Nicotine induces mitogen-activated protein kinase dependent vascular smooth muscle cell migration. *Atherosclerosis, 178*(2), 271-277. doi: 10.1016/j.atherosclerosis.2004.09.017

DiFranza, J. R., Aligne, C. A., & Weitzman, M. (2004). Prenatal and postnatal environmental tobacco smoke exposure and children's health. [Review]. *Pediatrics, 113*(4 Suppl), 1007-1015.

Diomede, L., Rognoni, P., Lavatelli, F., Romeo, M., del Favero, E., Cantu, L., . . . Merlini, G. (2014). A Caenorhabditis elegans-based assay recognizes immunoglobulin light chains causing heart amyloidosis. *Blood, 123*(23), 3543-3552. doi: 10.1182/blood-2013-10-525634

Domino , E. F., Hornbach , E., & Demana , T. (1993). The Nicotine Content of Common Vegetables. *New England Journal of Medicine, 329*(6), 437-437. doi: doi:10.1056/NEJM199308053290619

Dorn, G. W. (2013). MicroRNAs and the butterfly effect. *Cell Cycle, 12*(5), 707-708. doi: 10.4161/cc.23858

Dorn, G. W., 2nd. (2012). Decoding the cardiac message: the 2011 Thomas W. Smith Memorial Lecture. *Circ Res, 110*(5), 755-763. doi: 10.1161/CIRCRESAHA.111.256768

Dreyer, J. L. (2010). New insights into the roles of microRNAs in drug addiction and neuroplasticity. *Genome Med, 2*(12), 92. doi: 10.1186/gm213

Durazzo, T. C., Mattsson, N., & Weiner, M. W. (2014). Smoking and increased Alzheimer's disease risk: a review of potential mechanisms. *Alzheimers Dement, 10*(3 Suppl), S122-145. doi: 10.1016/j.jalz.2014.04.009

Eichhorn, S. W., Guo, H., McGeary, S. E., Rodriguez-Mias, R. A., Shin, C., Baek, D., . . . Bartel, D. P. (2014). mRNA destabilization is the dominant effect of mammalian microRNAs by the time substantial repression ensues. *Mol Cell, 56*(1), 104-115. doi: 10.1016/j.molcel.2014.08.028

Eipper-Mains, J. E., Eipper, B. A., & Mains, R. E. (2012). Global Approaches to the Role of miRNAs in Drug-Induced Changes in Gene Expression. *Front Genet, 3*, 109. doi: 10.3389/fgene.2012.00109

Eipper-Mains, J. E., Kiraly, D. D., Palakodeti, D., Mains, R. E., Eipper, B. A., & Graveley, B. R. (2011). microRNA-Seq reveals cocaine-regulated expression of striatal microRNAs. [Research Support, N.I.H., Extramural]. *RNA, 17*(8), 1529-1543. doi: 10.1261/rna.2775511

Epstein, H. F., & Benian, G. M. (2012). Paradigm shifts in cardiovascular research from Caenorhabditis elegans muscle. *Trends Cardiovasc Med, 22*(8), 201-209. doi: 10.1016/j.tcm.2012.07.021

Ewald, C. Y., & Li, C. (2009). Understanding the molecular basis of Alzheimer's disease using a Caenorhabditis elegans model system. *Brain Struct Funct, 214*(2-3), 263-283. doi: 10.1007/s00429-009-0235-3

Ey, J. L., Holberg, C. J., Aldous, M. B., Wright, A. L., Martinez, F. D., & Taussig, L. M. (1995). Passive smoke exposure and otitis media in the first year of life. Group Health Medical Associates. [Research Support, U.S. Gov't, P.H.S.]. *Pediatrics, 95*(5), 670-677.

Fabian, M. R., & Sonenberg, N. (2012). The mechanics of miRNA-mediated gene silencing: a look under the hood of miRISC. *Nat Struct Mol Biol, 19*(6), 586-593. doi: 10.1038/nsmb.2296

Fallingborg, J. (1999). Intraluminal pH of the human gastrointestinal tract. [Review]. *Dan Med Bull, 46*(3), 183-196.

Feng, Z., Li, W., Ward, A., Piggott, B. J., Larkspur, E. R., Sternberg, P. W., & Xu, X. Z. (2006). A *C. elegans* model of nicotine-dependent behavior: regulation by TRP-family channels. *Cell, 127*(3), 621-633. doi: 10.1016/j.cell.2006.09.035

Fiore, R., Khudayberdiev, S., Saba, R., & Schratt, G. (2011). MicroRNA function in the nervous system. [Review]. *Prog Mol Biol Transl Sci, 102*, 47-100. doi: 10.1016/B978-0-12-415795-8.00004-0

Fredriksson, A., & Archer, T. (2004). Neurobehavioural deficits associated with apoptotic neurodegeneration and vulnerability for ADHD. *Neurotox Res, 6*(6), 435-456.

Gartner A., Boag P. R., & B., B. T. (2008). Germline survival and apoptosis. WormBook: The *C. elegans* Research Community, Wormbook. Retrieved from http://www.wormbook.org. doi: 10.1895/wormbook.1.145.1.

Gedik, H., Erdal, M. E., Gorucu Yilmaz, S., Sengul, C., Sengul, C. B., & Herken, H. (2014). Association of microRNA Biogenesis Pathway Gene Variants and Alcohol Dependence Risk. *DNA Cell Biol, 34*(3), 220-226. doi: 10.1089/dna.2014.2549

Grando, S. A. (2014). Connections of nicotine to cancer. *Nat Rev Cancer, 14*(6), 419-429. doi: 10.1038/nrc3725

Guo, H., Ingolia, N. T., Weissman, J. S., & Bartel, D. P. (2010). Mammalian microRNAs predominantly act to decrease target mRNA levels. *Nature, 466*(7308), 835-840. doi: 10.1038/nature09267

Gyekis, J., Anthony, K., Foreman, J. E., Klein, L. C., & Vandenbergh, D. J. (2010). Perinatal nicotine exposure delays genital development in mice. *Reprod Toxicol, 29*(3), 378-380. doi: 10.1016/j.reprotox.2010.01.002

Ha, M., & Kim, V. N. (2014). Regulation of microRNA biogenesis. *Nat Rev Mol Cell Biol, 15*(8), 509-524. doi: 10.1038/nrm3838

Haberg, S. E., Stigum, H., Nystad, W., & Nafstad, P. (2007). Effects of pre- and postnatal exposure to parental smoking on early childhood respiratory health. [Research Support, Non-U.S. Gov't]. *Am J Epidemiol, 166*(6), 679-686. doi: 10.1093/aje/kwm134

Haugen, A. C., Schug, T. T., Collman, G., & Heindel, J. J. (2015). Evolution of DOHaD: the impact of environmental health sciences. *J Dev Orig Health Dis, 6*(2), 55-64. doi: 10.1017/S2040174414000580

Hernan, M. A., Takkouche, B., Caamano-Isorna, F., & Gestal-Otero, J. J. (2002). A meta-analysis of coffee drinking, cigarette smoking, and the risk of Parkinson's disease. [Meta-Analysis]. *Ann Neurol, 52*(3), 276-284. doi: 10.1002/ana.10277

Heusch, W. L., & Maneckjee, R. (1998). Signalling pathways involved in nicotine regulation of apoptosis of human lung cancer cells. [Research Support, U.S. Gov't, P.H.S.]. *Carcinogenesis, 19*(4), 551-556.

Hill, A. J., Mansfield, R., Lopez, J. M., Raizen, D. M., & Van Buskirk, C. (2014). Cellular Stress Induces a Protective Sleep-like State in *C. elegans*. *Curr Biol, 24*(20), 2399-2405. doi: 10.1016/j.cub.2014.08.040

Hill, J. (1761). *Cautions against the immoderate use of snuff: founded on the known qualities of the tobacco plant : and the effects it must produce when this way taken into the body : and enforced by instances of persons who have perished miserably of diseases, occasioned, or rendered incurable by its use* (2nd edition ed.). London: London : Printed for R. Baldwin in Pater-noster Row and J. Jackson in St. James-street, MDCCLXI [1761].

Hollander, J. A., Im, H. I., Amelio, A. L., Kocerha, J., Bali, P., Lu, Q., . . . Kenny, P. J. (2010). Striatal microRNA controls cocaine intake through CREB signalling. [Research Support, N.I.H., Extramural]. *Nature, 466*(7303), 197-202. doi: 10.1038/nature09202

Honda, Y., Honda, S., Narici, M., & Szewczyk, N. J. (2014). Spaceflight and ageing: reflecting on Caenorhabditis elegans in space. *Gerontology, 60*(2), 138-142. doi: 10.1159/000354772

Howitz, K. T., Bitterman, K. J., Cohen, H. Y., Lamming, D. W., Lavu, S., Wood, J. G., . . . Sinclair, D. A. (2003). Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan. *Nature, 425*(6954), 191-196. doi: 10.1038/nature01960

Hu, G., Yao, H., Chaudhuri, A. D., Duan, M., Yelamanchili, S. V., Wen, H., . . . Buch, S. (2012). Exosome-mediated shuttling of microRNA-29 regulates HIV Tat and morphine-mediated neuronal dysfunction. [Research Support, N.I.H., Extramural]. *Cell Death Dis, 3*, e381. doi: 10.1038/cddis.2012.114

Hu, Y., Matkovich, S. J., Hecker, P. A., Zhang, Y., Edwards, J. R., & Dorn, G. W., 2nd. (2012). Epitranscriptional orchestration of genetic reprogramming is an emergent property of stress-regulated cardiac microRNAs. *Proc Natl Acad Sci U S A, 109*(48), 19864-19869. doi: 10.1073/pnas.1214996109

Huang, H., Xu, Y., & van den Pol, A. N. (2011). Nicotine excites hypothalamic arcuate anorexigenic proopiomelanocortin neurons and orexigenic neuropeptide Y neurons: similarities and differences. *J Neurophysiol, 106*(3), 1191-1202. doi: 10.1152/jn.00740.2010

Huang, W., & Li, M. D. (2009). Nicotine modulates expression of miR-140*, which targets the 3'-untranslated region of dynamin 1 gene (Dnm1). [Research Support, N.I.H., Extramural]. *Int J Neuropsychopharmacol, 12*(4), 537-546. doi: 10.1017/S1461145708009528

Hubbard, E. J., & Greenstein, D. (2005). Introduction to the germ lineWormBook (2007/12/01 ed., pp. 1-4): The *C. elegans* Research Community, WormBook. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/18050415. doi: 10.1895/wormbook.1.18.1

Hubbard, E. J., Korta, D. Z., & Dalfo, D. (2013). Physiological control of germline development. *Adv Exp Med Biol, 757*, 101-131. doi: 10.1007/978-1-4614-4015-4_5

Hwang, J. W., Sundar, I. K., Yao, H., Sellix, M. T., & Rahman, I. (2014). Circadian clock function is disrupted by environmental tobacco/cigarette smoke, leading to lung inflammation and injury via a SIRT1-BMAL1 pathway. [Research Support, N.I.H., Extramural]. *FASEB J, 28*(1), 176-194. doi: 10.1096/fj.13-232629

Hwang, J. W., Yao, H., Caito, S., Sundar, I. K., & Rahman, I. (2013). Redox regulation of SIRT1 in inflammation and cellular senescence. *Free Radic Biol Med, 61*, 95-110. doi: 10.1016/j.freeradbiomed.2013.03.015

Ibanez-Ventoso, C., Vora, M., & Driscoll, M. (2008). Sequence relationships among *C. elegans*, D. melanogaster and human microRNAs highlight the extensive conservation of microRNAs in biology. [Research Support, N.I.H., Extramural]. *PLoS One, 3*(7), e2818. doi: 10.1371/journal.pone.0002818

Intskirveli, I., & Metherate, R. (2012). Nicotinic neuromodulation in auditory cortex requires MAPK activation in thalamocortical and intracortical circuits. [Research Support, N.I.H., Extramural]. *J Neurophysiol, 107*(10), 2782-2793. doi: 10.1152/jn.01129.2011

Iskandar, A. R., Liu, C., Smith, D. E., Hu, K. Q., Choi, S. W., Ausman, L. M., & Wang, X. D. (2013). beta-cryptoxanthin restores nicotine-reduced lung SIRT1 to normal levels and inhibits nicotine-promoted lung tumorigenesis and emphysema in A/J mice. *Cancer Prev Res (Phila), 6*(4), 309-320. doi: 10.1158/1940-6207.CAPR-12-0368

Jadhav, V., Hackl, M., Druz, A., Shridhar, S., Chung, C. Y., Heffner, K. M., . . . Borth, N. (2013). CHO microRNA engineering is growing up: recent successes and future challenges. *Biotechnol Adv, 31*(8), 1501-1513. doi: 10.1016/j.biotechadv.2013.07.007

James, W. H. (2013). Potential explanation of the reported association between maternal smoking and autism. *Environ Health Perspect, 121*(2), a42. doi: 10.1289/ehp.1206268

Janssen, H. L., Reesink, H. W., Lawitz, E. J., Zeuzem, S., Rodriguez-Torres, M., Patel, K., . . . Hodges, M. R. (2013). Treatment of HCV infection by targeting microRNA. *N Engl J Med, 368*(18), 1685-1694. doi: 10.1056/NEJMoa1209026

Kam, P. C., & Ferch, N. I. (2000). Apoptosis: mechanisms and clinical implications. [Review]. *Anaesthesia, 55*(11), 1081-1093.

Karpinski, J. P., Timpe, E. M., & Lubsch, L. (2010). Smoking cessation treatment for adolescents. *J Pediatr Pharmacol Ther, 15*(4), 249-263.

Kassie, F., Jarcho, M., & Endalew, A. (2010). Abstract PR-06: Upregulation of microRNA-21 (miR-21) in human bronchial epithelial cells chronically exposed to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) plus nicotine and modulation of these effects by diindolylmethane. *Cancer Prev Res, 3*(1 Suppl), A51. doi: 10.1158/1940-6207.PREV-09-PR-06

Kawakita A, Sato K, Makino H, Ikegami H, & Takayama S, e. a. (2008). Nicotine Acts on Growth Plate Chondrocytes to Delay Skeletal Growth through the α7 Neuronal Nicotinic Acetylcholine Receptor. *PLoS ONE, 3*(12), e3945. doi: doi:10.1371/journal.pone.0003945

Kichko, T. I., Lennerz, J., Eberhardt, M., Babes, R. M., Neuhuber, W., Kobal, G., & Reeh, P. W. (2013). Bimodal concentration-response of nicotine involves the nicotinic acetylcholine receptor, transient receptor potential vanilloid type 1, and transient receptor potential ankyrin 1 channels in mouse trachea and sensory neurons. [Research Support, Non-U.S. Gov't]. *J Pharmacol Exp Ther, 347*(2), 529-539. doi: 10.1124/jpet.113.205971

Kim, D. H., Saetrom, P., Snove, O., Jr., & Rossi, J. J. (2008). MicroRNA-directed transcriptional gene silencing in mammalian cells. *Proc Natl Acad Sci U S A, 105*(42), 16230-16235. doi: 10.1073/pnas.0808830105

Kim, V. N. (2005). MicroRNA biogenesis: coordinated cropping and dicing. *Nat Rev Mol Cell Biol, 6*(5), 376-385. doi: 10.1038/nrm1644

Kirienko, N. V., Mani, K., & Fay, D. S. (2010). Cancer models in *Caenorhabditis elegans*. *Dev Dyn, 239*(5), 1413-1448. doi: 10.1002/dvdy.22247

Kobet, R. A., Pan, X., Zhang, B., Pak, S. C., Asch, A. S., & Lee, M. H. (2014). : A Model System for Anti-Cancer Drug Discovery and Therapeutic Target Identification. [Review]. *Biomol Ther (Seoul), 22*(5), 371-383. doi: 10.4062/biomolther.2014.084

Kozomara, A., & Griffiths-Jones, S. (2014). miRBase: annotating high confidence microRNAs using deep sequencing data. [Research Support, Non-U.S. Gov't]. *Nucleic Acids Res, 42*(Database issue), D68-73. doi: 10.1093/nar/gkt1181

Kundu, P., Fabian, M. R., Sonenberg, N., Bhattacharyya, S. N., & Filipowicz, W. (2012). HuR protein attenuates miRNA-mediated repression by promoting miRISC dissociation from the target RNA. [Research Support, Non-U.S. Gov't]. *Nucleic Acids Res, 40*(11), 5088-5100. doi: 10.1093/nar/gks148

L'Hernault, S. W. (2006). Spermatogenesis. *WormBook, ed. The C. elegans Research Community, WormBook, doi/10.1895/wormbook.1.85.1, http://www.wormbook.org*.

Lee, H., Park, J. R., Yang, J., Kim, E., Hong, S. H., Woo, H. M., . . . Yang, S. R. (2014). Nicotine inhibits the proliferation by upregulation of nitric oxide and increased HDAC1 in mouse neural stem cells. [Research Support, Non-U.S. Gov't]. *In Vitro Cell Dev Biol Anim, 50*(8), 731-739. doi: 10.1007/s11626-014-9763-0

Lee, H. J. (2014). Additional stories of microRNAs. *Exp Biol Med (Maywood)*. doi: 10.1177/1535370214544269

Lee, R. C., Feinbaum, R. L., & Ambros, V. (1993). The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell, 75*(5), 843-854. doi: 0092-8674(93)90529-Y

Leung, A. K., & Sharp, P. A. (2010). MicroRNA functions in stress responses. *Mol Cell, 40*(2), 205-215. doi: 10.1016/j.molcel.2010.09.027

Li, C. Y., Mao, X., & Wei, L. (2008). Genes and (common) pathways underlying drug addiction. *PLoS Comput Biol, 4*(1), e2. doi: 10.1371/journal.pcbi.0040002

Li, M. D., & van der Vaart, A. D. (2011). MicroRNAs in addiction: adaptation's middlemen? *Mol Psychiatry, 16*(12), 1159-1168. doi: 10.1038/mp.2011.58

Li, X., & Kazgan, N. (2011). Mammalian sirtuins and energy metabolism. *Int J Biol Sci, 7*(5), 575-587.

Liang, H., Zhang, J., Zen, K., Zhang, C. Y., & Chen, X. (2013). Nuclear microRNAs and their unconventional role in regulating non-coding RNAs. *Protein Cell, 4*(5), 325-330. doi: 10.1007/s13238-013-3001-5

Liebrechts-Akkerman, G., Lao, O., Liu, F., van Sleuwen, B. E., Engelberts, A. C., L'Hoir M, P., . . . Kayser, M. (2011). Postnatal parental smoking: an important risk factor for SIDS. *Eur J Pediatr, 170*(10), 1281-1291. doi: 10.1007/s00431-011-1433-6

Lin, C., Yon, J. M., Jung, A. Y., Lee, J. G., Jung, K. Y., Kang, J. K., . . . Nam, S. Y. (2012). Resveratrol prevents nicotine-induced teratogenesis in cultured mouse embryos. [Research Support, Non-U.S. Gov't]. *Reprod Toxicol, 34*(3), 340-346. doi: 10.1016/j.reprotox.2012.05.097

Lopez-Bellido, R., Barreto-Valer, K., Sanchez-Simon, F. M., & Rodriguez, R. E. (2012). Cocaine modulates the expression of opioid receptors and miR-let-7d in zebrafish embryos. [Research Support, Non-U.S. Gov't]. *PLoS One, 7*(11), e50885. doi: 10.1371/journal.pone.0050885

Maegdefessel, L., Azuma, J., Toh, R., Deng, A., Merk, D. R., Raiesdana, A., . . . Tsao, P. S. (2012). MicroRNA-21 blocks abdominal aortic aneurysm development and nicotine-augmented expansion. *Sci Transl Med, 4*(122), 122ra122. doi: 10.1126/scitranslmed.3003441

Mango, S. E. (2007). The *C. elegans* pharynx: a model for organogenesis. *WormBook*, 1-26. doi: 10.1895/wormbook.1.129.1

Mantri, C. K., Pandhare Dash, J., Mantri, J. V., & Dash, C. C. (2012). Cocaine enhances HIV-1 replication in CD4+ T cells by down-regulating MiR-125b. *PLoS One, 7*(12), e51387. doi: 10.1371/journal.pone.0051387

Marczylo, E. L., Amoako, A. A., Konje, J. C., Gant, T. W., & Marczylo, T. H. (2012). Smoking induces differential miRNA expression in human spermatozoa: a potential transgenerational epigenetic concern? [Research Support, Non-U.S. Gov't]. *Epigenetics, 7*(5), 432-439. doi: 10.4161/epi.19794

Martins-Green, M., Adhami, N., Frankos, M., Valdez, M., Goodwin, B., Lyubovitsky, J., . . . Curras-Collazo, M. (2014). Cigarette Smoke Toxins Deposited on Surfaces: Implications for Human Health. *PLoS One, 9*(1), e86391. doi: 10.1371/journal.pone.0086391

Mathonnet, G., Fabian, M. R., Svitkin, Y. V., Parsyan, A., Huck, L., Murata, T., . . . Sonenberg, N. (2007). MicroRNA inhibition of translation initiation in vitro by targeting the cap-binding complex eIF4F. *Science, 317*(5845), 1764-1767. doi: 10.1126/science.1146067

Matkovich, S. J. (2014). MicroRNAs in the Stressed Heart: Sorting the Signal from the Noise. [Review]. *Cells, 3*(3), 778-801. doi: 10.3390/cells3030778

Maupus, E. (1990). Modes et formes de reproduction des nematodes *Exp. Gen., 8*, 463-624.

McGary, K. L., Park, T. J., Woods, J. O., Cha, H. J., Wallingford, J. B., & Marcotte, E. M. (2010). Systematic discovery of nonobvious human disease models through orthologous phenotypes. *Proc Natl Acad Sci U S A, 107*(14), 6544-6549. doi: 10.1073/pnas.0910200107

McGhee, J. D. (2007). The *C. elegans* intestine. *WormBook*, 1-36. doi: 10.1895/wormbook.1.133.1

McGraw-Hill-Higher-Education. (2011). Caenorhabditis elegans: Genetic Portrait of a Simple Multicellular Animal. [WWW document] URL: http://highered.mcgraw-hill.com/sites/dl/free/007352526x/387004/Reference_C.pdf

McNeill, E., & Van Vactor, D. (2012). MicroRNAs shape the neuronal landscape. *Neuron, 75*(3), 363-379. doi: 10.1016/j.neuron.2012.07.005

Medina, P. P., Nolde, M., & Slack, F. J. (2010). OncomiR addiction in an in vivo model of microRNA-21-induced pre-B-cell lymphoma. [Research Support, Non-U.S. Gov't]. *Nature, 467*(7311), 86-90. doi: 10.1038/nature09284

Mineur, Y. S., Abizaid, A., Rao, Y., Salas, R., DiLeone, R. J., Gundisch, D., . . . Picciotto, M. R. (2011). Nicotine decreases food intake through activation of POMC neurons. *Science, 332*(6035), 1330-1332. doi: 10.1126/science.1201889

Miska, E., Alvarez-Saavedra, E., Abbott, A., Lau, N., Hellman, A., & al., e. (2007). Most Caenorhabditis elegans microRNAs are individually not essential for development or viability. *PLoS Genet 3*. doi: e215. doi:10.1371/journal.pgen.0030215. doi: 10.1371/journal.pgen.0030215

Mitchell, P. H., Bull, K., Glautier, S., Hopper, N. A., Holden-Dye, L., & O'Connor, V. (2007). The concentration-dependent effects of ethanol on Caenorhabditis elegans behaviour. [Research Support, Non-U.S. Gov't]. *Pharmacogenomics J, 7*(6), 411-417. doi: 10.1038/sj.tpj.6500440

Nakagawa, T., & Guarente, L. (2011). Sirtuins at a glance. *J Cell Sci, 124*(Pt 6), 833-838. doi: 10.1242/jcs.081067

Napuri, J., Pilakka-Kanthikeel, S., Raymond, A., Agudelo, M., Yndart-Arias, A., Saxena, S. K., & Nair, M. (2013). Cocaine enhances HIV-1 infectivity in monocyte derived dendritic cells by suppressing microRNA-155. [Research Support, N.I.H., Extramural]. *PLoS One, 8*(12), e83682. doi: 10.1371/journal.pone.0083682

Ng, T. K., Carballosa, C. M., Pelaez, D., Wong, H. K., Choy, K. W., Pang, C. P., & Cheung, H. S. (2013). Nicotine Alters MicroRNA Expression and Hinders Human Adult Stem Cell Regenerative Potential. *Stem Cells Dev., 22*(5), 781-790. doi: 10.1089/scd.2012.0434

NIDA. (2014). Dr. Joni Rutter to lead NIDA's genetics and basic science research division. Retrieved from http://www.drugabuse.gov/news-events/news-releases/2014/05/dr-joni-rutter-to-lead-nidas-genetics-basic-science-research-division on December 10, 2014.

Nielsen, H. M., & Rassing, M. R. (2002). Nicotine permeability across the buccal TR146 cell culture model and porcine buccal mucosa in vitro: effect of pH and concentration. *Eur J Pharm Sci, 16*(3), 151-157.

Nyayanit, D., & Gadgil, C. J. (2015). Mathematical modeling of combinatorial regulation suggests that apparent positive regulation of targets by miRNA could be an artifact resulting from competition for mRNA. *RNA*. doi: 10.1261/rna.046862.114

Owen, M. J., Baldwin, C. D., Swank, P. R., Pannu, A. K., Johnson, D. L., & Howie, V. M. (1993). Relation of infant feeding practices, cigarette smoke exposure, and group child care to the onset and duration of otitis media with effusion in the first two years of life. [Research Support, U.S. Gov't, P.H.S.]. *J Pediatr, 123*(5), 702-711.

Park, G. J., Kim, Y. S., Kang, K. L., Bae, S. J., Baek, H. S., Auh, Q. S., . . . Kim, E. C. (2013). Effects of sirtuin 1 activation on nicotine and lipopolysaccharide-induced cytotoxicity and inflammatory cytokine production in human gingival fibroblasts. [Research Support, Non-U.S. Gov't]. *J Periodontal Res, 48*(4), 483-492. doi: 10.1111/jre.12030

Pfeffer, S., Sewer, A., Lagos-Quintana, M., Sheridan, R., Sander, C., Grasser, F. A., . . . Tuschl, T. (2005). Identification of microRNAs of the herpesvirus family. *Nat Methods, 2*(4), 269-276. doi: 10.1038/nmeth746

Pietrzykowski, A. Z. (2010). The role of microRNAs in drug addiction: a big lesson from tiny molecules. [Review]. *Int Rev Neurobiol, 91*, 1-24. doi: 10.1016/S0074-7742(10)91001-5

Pietrzykowski, A. Z., Friesen, R. M., Martin, G. E., Puig, S. I., Nowak, C. L., Wynne, P. M., . . . Treistman, S. N. (2008). Posttranscriptional regulation of BK channel splice variant stability by miR-9 underlies neuroadaptation to alcohol. *Neuron, 59*(2), 274-287. doi: 10.1016/j.neuron.2008.05.032

Pilon, M. (2014). Developmental genetics of the Caenorhabditis elegans pharynx. [Research Support, Non-U.S. Gov't]. *Wiley Interdiscip Rev Dev Biol, 3*(4), 263-280. doi: 10.1002/wdev.139

Potera, C. (2010). Smoking and secondhand smoke. Study finds no level of SHS exposure free of effects. [News]. *Environ Health Perspect, 118*(11), A474.

Prins, S. A., Przybycien-Szymanska, M. M., Rao, Y. S., & Pak, T. R. (2014). Long-term effects of peripubertal binge EtOH exposure on hippocampal microRNA expression in the rat. [Research Support, N.I.H., Extramural]. *PLoS One, 9*(1), e83166. doi: 10.1371/journal.pone.0083166

Rajasethupathy, P., Fiumara, F., Sheridan, R., Betel, D., Puthanveettil, S. V., Russo, J. J., . . . Kandel, E. (2009). Characterization of small RNAs in Aplysia reveals a role for miR-124 in constraining synaptic plasticity through CREB. *Neuron, 63*(6), 803-817. doi: 10.1016/j.neuron.2009.05.029

Rayner, K. J., & Hennessy, E. J. (2013). Extracellular communication via microRNA: lipid particles have a new message. [Review]. *J Lipid Res, 54*(5), 1174-1181. doi: 10.1194/jlr.R034991

Rickles, D., Hawe, P., & Shiell, A. (2007). A simple guide to chaos and complexity. *J Epidemiol Community Health, 61*(11), 933-937. doi: 10.1136/jech.2006.054254

Riddle, D. L., Blumenthal, T., & B.J., M. (1997). C. elegans II. 2nd edition. Retrieved from http://www.ncbi.nlm.nih.gov/books/NBK20086/

Riera, C. E., Huising, M. O., Follett, P., Leblanc, M., Halloran, J., Van Andel, R., . . . Dillin, A. (2014). TRPV1 pain receptors regulate longevity and metabolism by neuropeptide

signaling. [Research Support, Non-U.S. Gov't]. *Cell, 157*(5), 1023-1036. doi: 10.1016/j.cell.2014.03.051
Ripoli, A., Rainaldi, G., Rizzo, M., Mercatanti, A., & Pitto, L. (2010). The Fuzzy Logic of MicroRNA Regulation: A Key to Control Cell Complexity. *Curr Genomics, 11*(5), 350-353. doi: 10.2174/138920210791616707
Roberts, T. C. (2014). The MicroRNA Biology of the Mammalian Nucleus. *Mol Ther Nucleic Acids, 3*, e188. doi: 10.1038/mtna.2014.40
Rubinstein, M., & Low, M. J. (2011). The smoking gun in nicotine-induced anorexia. *Cell Metab, 14*(2), 145-147. doi: 10.1016/j.cmet.2011.07.006
Ruiz-Lancheros, E., Viau, C., Walter, T. N., Francis, A., & Geary, T. G. (2011). Activity of novel nicotinic anthelmintics in cut preparations of Caenorhabditis elegans. [Research Support, Non-U.S. Gov't]. *Int J Parasitol, 41*(3-4), 455-461. doi: 10.1016/j.ijpara.2010.11.009
Saba, R., Storchel, P. H., Aksoy-Aksel, A., Kepura, F., Lippi, G., Plant, T. D., & Schratt, G. M. (2012). Dopamine-regulated microRNA MiR-181a controls GluA2 surface expression in hippocampal neurons. *Mol Cell Biol, 32*(3), 619-632. doi: 10.1128/MCB.05896-11
Sathyan, P., Golden, H. B., & Miranda, R. C. (2007). Competing interactions between micro-RNAs determine neural progenitor survival and proliferation after ethanol exposure: evidence from an ex vivo model of the fetal cerebral cortical neuroepithelium. *J Neurosci, 27*(32), 8546-8557. doi: 10.1523/JNEUROSCI.1269-07.2007
Schaedel, O. N., Gerisch, B., Antebi, A., & Sternberg, P. W. (2012). Hormonal signal amplification mediates environmental conditions during development and controls an irreversible commitment to adulthood. *PLoS Biol, 10*(4), e1001306. doi: 10.1371/journal.pbio.1001306
Schaefer, A., Im, H. I., Veno, M. T., Fowler, C. D., Min, A., Intrator, A., . . . Greengard, P. (2010). Argonaute 2 in dopamine 2 receptor-expressing neurons regulates cocaine addiction. *J Exp Med, 207*(9), 1843-1851. doi: 10.1084/jem.20100451
Sellings, L., Pereira, S., Qian, C., Dixon-McDougall, T., Nowak, C., Zhao, B., . . . van der Kooy, D. (2013). Nicotine-motivated behavior in Caenorhabditis elegans requires the nicotinic acetylcholine receptor subunits acr-5 and acr-15. *Eur J Neurosci, 37*(5), 743-756. doi: 10.1111/ejn.12099
Shan, H., Zhang, Y., Lu, Y., Pan, Z., Cai, B., Wang, N., . . . Yang, B. (2009). Downregulation of miR-133 and miR-590 contributes to nicotine-induced atrial remodelling in canines. [Research Support, Non-U.S. Gov't]. *Cardiovasc Res., 83*(3), 465-472. doi: 10.1093/cvr/cvp130
Shaye, D. D., & Greenwald, I. (2011). OrthoList: a compendium of *C. elegans* genes with human orthologs. *PLoS One, 6*(5), e20085. doi: 10.1371/journal.pone.0020085
Shi, D., Guo, W., Chen, W., Fu, L., Wang, J., Tian, Y., . . . Deng, W. (2012). Nicotine promotes proliferation of human nasopharyngeal carcinoma cells by regulating alpha7AChR, ERK, HIF-1alpha and VEGF/PEDF signaling. [Research Support, Non-U.S. Gov't]. *PLoS One, 7*(8), e43898. doi: 10.1371/journal.pone.0043898
Shin, V. Y., Jin, H., Ng, E. K., Cheng, A. S., Chong, W. W., Wong, C. Y., . . . Chu, K. M. (2011). NF-kappaB targets miR-16 and miR-21 in gastric cancer: involvement of prostaglandin E receptors. [Research Support, Non-U.S. Gov't]. *Carcinogenesis, 32*(2), 240-245. doi: 10.1093/carcin/bgq240

Smith, A. M., Dwoskin, L. P., & Pauly, J. R. (2010). Early exposure to nicotine during critical periods of brain development: Mechanisms and consequences. *J Pediatr Biochem, 1*(2), 125-141. doi: 10.3233/JPB-2010-0012

Song, B.-m., & Avery, L. (2013). The pharynx of the nematode *C. elegans*. *Worm, 2*(1), e21833. doi: 10.4161/worm.21833

Soule, E. K., Lipato, T., & Eissenberg, T. (2015). Waterpipe tobacco smoking: A new smoking epidemic among the young? *Curr Pulmonol Rep, 4*(4), 163-172. doi: 10.1007/s13665-015-0124-6

Steiner, R. C., Heath, C. J., & Picciotto, M. R. (2007). Nicotine-induced phosphorylation of ERK in mouse primary cortical neurons: evidence for involvement of glutamatergic signaling and CaMKII. [Research Support, N.I.H., Extramural]. *J Neurochem, 103*(2), 666-678. doi: 10.1111/j.1471-4159.2007.04799.x

Strachan, D. P., & Cook, D. G. (1998). Health effects of passive smoking. 4. Parental smoking, middle ear disease and adenotonsillectomy in children. *Thorax, 53*(1), 50-56.

Stroynowska-Czerwinska, A., Fiszer, A., & Krzyzosiak, W. J. (2014). The panorama of miRNA-mediated mechanisms in mammalian cells. *Cell Mol Life Sci, 71*(12), 2253-2270. doi: 10.1007/s00018-013-1551-6

Sugano, T., Yanagita, T., Yokoo, H., Satoh, S., Kobayashi, H., & Wada, A. (2006). Enhancement of insulin-induced PI3K/Akt/GSK-3beta and ERK signaling by neuronal nicotinic receptor/PKC-alpha/ERK pathway: up-regulation of IRS-1/-2 mRNA and protein in adrenal chromaffin cells. *J Neurochem, 98*(1), 20-33. doi: 10.1111/j.1471-4159.2006.03846.x

Svoboda, K. R., Vijayaraghavan, S., & Tanguay, R. L. (2002). Nicotinic receptors mediate changes in spinal motoneuron development and axonal pathfinding in embryonic zebrafish exposed to nicotine. *J Neurosci, 22*(24), 10731-10741.

Takami, K., Saito, H., Okuda, M., Takano, M., & Inui, K. I. (1998). Distinct characteristics of transcellular transport between nicotine and tetraethylammonium in LLC-PK1 cells. [Research Support, Non-U.S. Gov't]. *J Pharmacol Exp Ther, 286*(2), 676-680.

Taki, F., Pan, X., & Zhang, B. (2013). Nicotine Exposure Caused Significant Transgenerational Heritable Behavioral Changes in Caenorhabditis elegans. *Excli J., 12*(1611-2156), 793-806. doi: http://www.excli.de/vol12/Zhang_10092013_proof.pdf

Taki, F. A., Pan, X., Lee, M.-H., & Zhang, B. (2014). Nicotine exposure and transgenerational impact: a prospective study on small regulatory microRNAs. *Sci. Rep., 4*(7513). doi: DOI:10.1038/srep07513

Taki, F. A., Pan, X., & Zhang, B. (2013). Chronic nicotine exposure systemically alters microRNA expression profiles during post-embryonic stages in Caenorhabditis elegans. *J Cell Physiol, 229*(1), 79-89. doi: 10.1002/jcp.24419

Taki, F. A., Pan, X., & Zhang, B. (2015). Revisiting Chaos Theorem to Understand the Nature of Mirnas in Response to Drugs of Abuse. *J Cell Physiol*. doi: 10.1002/jcp.25037

Tang, R., Li, L., Zhu, D., Hou, D., Cao, T., Gu, H., . . . Zen, K. (2012). Mouse miRNA-709 directly regulates miRNA-15a/16-1 biogenesis at the posttranscriptional level in the nucleus: evidence for a microRNA hierarchy system. [Research Support, Non-U.S. Gov't]. *Cell Res, 22*(3), 504-515. doi: 10.1038/cr.2011.137

Tatro, E. T., Hefler, S., Shumaker-Armstrong, S., Soontornniyomkij, B., Yang, M., Yermanos, A., . . . Achim, C. L. (2013). Modulation of BK channel by MicroRNA-9 in neurons after

exposure to HIV and methamphetamine. *J Neuroimmune Pharmacol, 8*(5), 1210-1223. doi: 10.1007/s11481-013-9446-8

Toborek, M., Son, K. W., Pudelko, A., King-Pospisil, K., Wylegala, E., & Malecki, A. (2007). ERK 1/2 signaling pathway is involved in nicotine-mediated neuroprotection in spinal cord neurons. [Research Support, Non-U.S. Gov't]. *J Cell Biochem, 100*(2), 279-292. doi: 10.1002/jcb.21013

Tsai, J. R., Chong, I. W., Chen, C. C., Lin, S. R., Sheu, C. C., & Hwang, J. J. (2006). Mitogen-activated protein kinase pathway was significantly activated in human bronchial epithelial cells by nicotine. [Research Support, Non-U.S. Gov't]. *DNA Cell Biol, 25*(5), 312-322. doi: 10.1089/dna.2006.25.312

van der Velde, K. J., de Haan, M., Zych, K., Arends, D., Snoek, L. B., Kammenga, J. E., . . . Li, Y. (2014). WormQTLHD--a web database for linking human disease to natural variation data in *C. elegans*. [Research Support, Non-U.S. Gov't]. *Nucleic Acids Res, 42*(Database issue), D794-801. doi: 10.1093/nar/gkt1044

Vidigal, J. A., & Ventura, A. (2014). The biological functions of miRNAs: lessons from in vivo studies. [Review]. *Trends Cell Biol, 25*(3), 137-147. doi: 10.1016/j.tcb.2014.11.004

Voisine, C., Varma, H., Walker, N., Bates, E. A., Stockwell, B. R., & Hart, A. C. (2007). Identification of potential therapeutic drugs for huntington's disease using Caenorhabditis elegans. *PLoS One, 2*(6), e504. doi: 10.1371/journal.pone.0000504

Wang, L., Li, X., Zhou, Y., Shi, H., Xu, C., He, H., . . . Shan, H. (2014). Downregulation of miR-133 via MAPK/ERK signaling pathway involved in nicotine-induced cardiomyocyte apoptosis. [Research Support, Non-U.S. Gov't]. *Naunyn Schmiedebergs Arch Pharmacol, 387*(2), 197-206. doi: 10.1007/s00210-013-0929-1

Ward, S., & Carrel, J. S. (1979). Fertilization and sperm competition in the nematodeCaenorhabditis elegans. *Developmental Biology, 73*(2), 304-321.

Wei, H., Alberts, I., & Li, X. (2014). The apoptotic perspective of autism. *Int J Dev Neurosci, 36*, 13-18. doi: 10.1016/j.ijdevneu.2014.04.004

WHO. (2007). Smoke-free inside. *World Health Organization. Retrieved on 12/12/2014 from http://www.who.int/tobacco/wntd/2007/wntd_2007_brochure.pdf*.

WHO. (2013). World Heath Statistics 2013. *World Health Organization. Retrieved on March.19.2014, from http://www.who.int/gho/publications/world_health_statistics/EN_WHS2013_Part3.pdf?ua=1*

Wilczynska, A., & Bushell, M. (2015). The complexity of miRNA-mediated repression. [Review]. *Cell Death Differ, 22*(1), 22-33. doi: 10.1038/cdd.2014.112

Wu, Q., Hwang, C. K., Zheng, H., Wagley, Y., Lin, H. Y., Kim do, K., . . . Wei, L. N. (2013). MicroRNA 339 down-regulates mu-opioid receptor at the post-transcriptional level in response to opioid treatment. *FASEB J, 27*(2), 522-535. doi: 10.1096/fj.12-213439

Wu, Q., Zhang, L., Law, P. Y., Wei, L. N., & Loh, H. H. (2009). Long-term morphine treatment decreases the association of mu-opioid receptor (MOR1) mRNA with polysomes through miRNA23b. *Mol Pharmacol, 75*(4), 744-750. doi: 10.1124/mol.108.053462

Yang, S. R., Wright, J., Bauter, M., Seweryniak, K., Kode, A., & Rahman, I. (2007). Sirtuin regulates cigarette smoke-induced proinflammatory mediator release via RelA/p65 NF-kappaB in macrophages in vitro and in rat lungs in vivo: implications for chronic inflammation and aging. [Research Support, N.I.H., Extramural]. *Am J Physiol Lung Cell Mol Physiol, 292*(2), L567-576. doi: 10.1152/ajplung.00308.2006

Zadeh, L. A. (1973). Outline of a New Approach to the Analysis of Complex Systems and Decision Processes. *Systems, Man and Cybernetics, IEEE Transactions on, SMC-3*(1), 28-44. doi: 10.1109/tsmc.1973.5408575

Zamora-Martinez, E. R., & Edwards, S. (2014). Neuronal extracellular signal-regulated kinase (ERK) activity as marker and mediator of alcohol and opioid dependence. [Review]. *Front Integr Neurosci, 8*, 24. doi: 10.3389/fnint.2014.00024

Zeidler, R., Albermann, K., & Lang, S. (2007). Nicotine and apoptosis. [Review]. *Apoptosis, 12*(11), 1927-1943. doi: 10.1007/s10495-007-0102-8

Zhang, B., Wang, Q., & Pan, X. (2007). MicroRNAs and their regulatory roles in animals and plants. [Review]. *J Cell Physiol., 210*(2), 279-289. doi: 10.1002/jcp.20869

Zheng, S. Q., Ding, A. J., Li, G. P., Wu, G. S., & Luo, H. R. (2013). Drug absorption efficiency in Caenorhbditis elegans delivered by different methods. [Research Support, Non-U.S. Gov't]. *PLoS One, 8*(2), e56877. doi: 10.1371/journal.pone.0056877

Zhou, S., Rosenthal, D. G., Sherman, S., Zelikoff, J., Gordon, T., & Weitzman, M. (2014). Physical, behavioral, and cognitive effects of prenatal tobacco and postnatal secondhand smoke exposure. [Review]. *Curr Probl Pediatr Adolesc Health Care, 44*(8), 219-241. doi: 10.1016/j.cppeds.2014.03.007

Zhu, G., Zhang, F., & Li, W. (2014). Nematodes feel a craving--using Caenorhabditis elegans as a model to study alcohol addiction. [Research Support, Non-U.S. Gov't]. *Neurosci Bull, 30*(4), 595-600. doi: 10.1007/s12264-014-1451-7

Table 1.1: The amount of nicotine in common vegetables (Domino et al., 1993).

VEGETABLE	HIGHEST REPORTED MEAN NICOTINE CONTENT	REFERENCE	AMOUNT OF VEGETABLE REQUIRED TO OBTAIN 1 μg OF NICOTINE*
	ng/g		*g*
Cauliflower	16.8	Davis et al.[4]	59.5
Cauliflower	3.8	Present study	263.4
Eggplant	100.0	Castro and Monji[2]	10.0
Potato peel	4.8	Davis et al.[4]	208.0
Potato pulp	15.3	Davis et al.[4]	65.4
Potatoes	7.1	Present study	140.4
Green tomatoes	42.8	Castro and Monji[2]	23.4
Pureed tomatoes	52.0	Castro and Monji[2]	19.2
Ripe tomatoes	4.3	Castro and Monji[2]	233.0
Ripe tomatoes	4.1	Present study	244.0
Tomatoes	10.7	Sheen[3]	93.5

*One microgram of nicotine is the amount a passive smoker would absorb in about three hours in a room with a minimal amount of tobacco smoke.

Table 1.2: Pro-apoptotic impact of nicotine on different biological systems

Model system	Exposure period	Tissue affected	Mechanism for apoptosis	Physiological impact	References
Wistar rats	Fetus and neonates: 2 weeks prior to mating until weaning	Pancreatic beta-islet cells	Mitochondrial oxidative stress	Postnatal dysglycemia and obesity. (diabetes)	(Bruin, Gerstein, et al., 2008; Bruin et al., 2007)
Healthy human molars	48 hrs	Human periodontal ligament stem cell	nAChR α7 and β4 Subunits p53	Periodontal disease	(S. Y. Kim, Kang, Lee, & Heo, 2012)
Mice	8 weeks mice: 5 hours/day, 5 days/week for 6 months	Contralateral eye	Oxidative damage to retinal pigmented epithelium (RPE)	Age-related macular degeneration (AMD)	(Fujihara M, Nagai N, Sussan TE, Biswal S, & JT, 2008)
Wistar rats	0.6 mg/kg for 21 days	Pulmonary epithelial cells	Oxidative stress	Lung injury	(Demiralay et al., 2006)
Wistar rats (1-3 days old)	48 hr treatment to the cell culture	Primary cultures of neonatal rat cardiomyocytes	ERK1/2–serum response factor (SRF)–miR-133 signaling	Cardiovascular disease	(L. Wang et al., 2014)
Smokers lung tissue with pulmonary emphysema, mouse, rat, and human AMs, and human blood monocyte-derived macrophages	..	Alveolar macrophages	Increased oxidative stress, mitochondrial dysfunction, p-53 and Fas independent	Macrophage dysfunction and lung disease	(Aoshiba, Yasui, & Nagai, 2000)
Normal lung biopsies	3-48 hrs	Primary human lung fibroblasts	Oxidative stress	Lung disorders like emphysema	(Baglole et al., 2006)

Table 1.3: A list of miRNAs reported to be upregulated in at least two DA (F. A. Taki et al., 2015).

Drugs	total	microRNAs
Alcohol, Anesthetics, Opioids, Stimulants, Tobacco/nicotine	1	let-7
Alcohol, Cannabinoids, Stimulants, Tobacco/nicotine	4	miR-152 miR-27a miR-125b miR-194
Alcohol, Opioids, Stimulants, Tobacco/nicotine	2	miR-9 miR-1
Alcohol, Anesthetics, Stimulants, Tobacco/nicotine	2	miR-212 miR-328
Alcohol, Cannabinoids, Opioids, Tobacco/nicotine	4	miR-335 miR-29c miR-21 miR-15b
Alcohol, Anesthetics, Opioids, Tobacco/nicotine	1	miR-132
Alcohol, Anesthetics, Opioids, Stimulants	1	miR-124
Alcohol, Anesthetics, Cannabinoids, Opioids	1	miR-29b
Alcohol, Stimulants, Tobacco/nicotine	6	miR-199a-3p miR-18a miR-196b miR-26b miR-542-3p miR-451
Alcohol, Cannabinoids, Tobacco/nicotine	5	miR-221 miR-642 miR-19b miR-24 miR-141
Alcohol, Opioids, Tobacco/nicotine	5	miR-192 miR-137 miR-365 miR-7 miR-29a
Alcohol, Anesthetics, Tobacco/nicotine	3	miR-222 miR-92a miR-301a
Alcohol, Cannabinoids, Stimulants	4	miR-665 miR-22

Table 1.3: A list of miRNAs reported to be upregulated in at least two DA (F. A. Taki et al., 2015).

Drugs	total	microRNAs
		miR-431 miR-27b
Alcohol, Anesthetics, Stimulants	3	miR-375 miR-144 miR-34b
Alcohol, Anesthetics, Cannabinoids	1	miR-139-5p
Cannabinoids, Stimulants, Tobacco/nicotine	2	miR-218 miR-135a
Cannabinoids, Opioids, Tobacco/nicotine	2	miR-20a miR-224
Anesthetics, Opioids, Tobacco/nicotine	1	miR-211
Anesthetics, Cannabinoids, Stimulants	1	miR-376a
Alcohol, Tobacco/nicotine	18	miR-188 miR-503 miR-351 miR-139-3p miR-200c miR-26a miR-30e miR-181b miR-203 miR-30d miR-93 miR-132_St miR-135b miR-374b miR-18a_St miR-27a_St miR-25 miR-140_St
Alcohol, Stimulants	11	miR-153 miR-125b-3p miR-708 miR-523 miR-369-5p miR-28_St miR-196a miR-32 miR-155

Table 1.3: A list of miRNAs reported to be upregulated in at least two DA (F. A. Taki et al., 2015).

Drugs	total	microRNAs
		miR-130b
		miR-181a
Alcohol, Cannabinoids	21	miR-93_St
		miR-125a-3p
		miR-200a
		miR-376c
		miR-145
		miR-339-5p
		miR-379
		miR-10b
		miR-370
		miR-183
		miR-323-3p
		miR-322
		miR-10a
		miR-26b_St
		miR-30e-3p
		miR-101
		miR-149
		miR-215
		miR-99a
		miR-130a
		miR-1195
Alcohol, Opioids	4	miR-103
		miR-122
		miR-302b
		miR-107
Alcohol, Anesthetics	10	miR-136-5p
		miR-181a-5p
		miR-34a
		miR-146a
		miR-221-3p
		miR-124-5p
		miR-214
		miR-488-3p
		miR-138-5p
		miR-30e-5p
Stimulants, Tobacco/nicotine	7	miR-30c
		miR-126_St
		miR-223

Table 1.3: A list of miRNAs reported to be upregulated in at least two DA (F. A. Taki et al., 2015).

Drugs	total	microRNAs
		miR-148a miR-15a miR-331-3p miR-16
Cannabinoids, Tobacco/nicotine	7	miR-186 miR-183_St miR-106b miR-362-3p miR-96 miR-23a miR-543
Anesthetics, Tobacco/nicotine	1	miR-9_St
Cannabinoids, Stimulants	4	miR-383 miR-409-3p miR-20a_St miR-374
Anesthetics, Stimulants	2	miR-34c miR-129
Cannabinoids, Opioids	5	miR-142-3p miR-499 miR-154 miR-331 miR-539
Anesthetics, Cannabinoids	2	miR-487b miR-495
Anesthetics, Opioids	1	miR-339-3p

Table 1.4: A list of miRNAs reported to be downregulated in at least two DA (F. A. Taki et al., 2015).

Drugs	total	microRNA(s)
Alcohol, Anesthetics, Stimulants, Tobacco/nicotine	2	miR-200a miR-200b
Alcohol, Stimulants, Tobacco/nicotine	6	miR-192 miR-128a miR-223 miR-124a miR-99a let-7
Alcohol, Cannabinoids, Tobacco/nicotine	2	miR-210 miR-374
Alcohol, Anesthetics, Tobacco/nicotine	2	miR-152 miR-10b
Alcohol, Cannabinoids, Stimulants	1	miR-335-5p
Alcohol, Opioids, Stimulants	3	miR-181b miR-382 miR-155
Alcohol, Anesthetics, Stimulants	2	miR-183 miR-200c
Alcohol, Cannabinoids, Opioids	1	miR-182
Alcohol, Anesthetics, Opioids	1	miR-296
Opioids, Stimulants, Tobacco/nicotine	2	miR-150 miR-125b
Anesthetics, Stimulants, Tobacco/nicotine	1	miR-429
Alcohol, Tobacco/nicotine	21	miR-342-5p miR-193b miR-98 miR-100 miR-30a miR-30a-5p miR-101a miR-362 miR-30C miR-19b miR-335 miR-29c miR-21 miR-19a miR-544

Table 1.4: A list of miRNAs reported to be downregulated in at least two DA (F. A. Taki et al., 2015).

Drugs	total	microRNA(s)
		miR-186 miR-352 miR-301a miR-622 miR-26a miR-449b
Alcohol, Stimulants	6	miR-212 miR-29b miR-154 miR-124 miR-181a miR-127
Alcohol, Cannabinoids	4	miR-499 miR-324-5p miR-27a_St miR-10b_St
Alcohol, Opioids	3	miR-606 miR-572 miR-184
Alcohol, Anesthetics	3	miR-203 miR-206 miR-871
Stimulants, Tobacco/nicotine	3	miR-31 miR-99b_St miR-125a-5p
Cannabinoids, Tobacco/nicotine	6	miR-762 miR-223_St miR-708 miR-423-5p miR-500 miR-1226_St
Opioids, Tobacco/nicotine	4	miR-1915 miR-99b miR-146a miR-149
Anesthetics, Tobacco/nicotine	2	miR-224 miR-1
Cannabinoids, Stimulants	1	miR-27a

Table 1.4: A list of miRNAs reported to be downregulated in at least two DA (F. A. Taki et al., 2015).

Drugs	total	microRNA(s)
Opioids, Stimulants	3	miR-28 miR-410 miR-133b
Anesthetics, Stimulants	6	miR-144 miR-451 miR-143 miR-199a-5p miR-34c miR-370
Anesthetics, Cannabinoids	1	miR-340-3p
Anesthetics, Opioids	1	miR-378

Table 1.5: A summary of 118 studies on miRNAs in response to seven categories of drugs of abuse (F. A. Taki et al., 2015).

https://docs.google.com/spreadsheets/d/1_Kk_CQS-MA2CunMNHaQcOh_VsspURqdrnBo29Ptm9vo/edit#gid=0

Table 1.6: *C. elegans* lifecycle (Byerly, Cassada, & Russell, 1976).

DEVELOPMENT AT DIFFERENT TEMPERATURES

	"16°C" (16.0 ± 0.3°C)	"20°C" (19.5 ± 0.5°C)	"25°C" (25.0 ± 0.2°C)
Egg laid	0 hr	0 hr	0 hr
Egg hatches	16-18 hr	10-12 hr	8-9 hr
First-molt lethargus	36.5 hr	26 hr	18.0 hr
Second-molt lethargus	48 hr	34.5 hr	25.5 hr
Third-molt lethargus	60.0 hr	43.5 hr	31 hr
Fourth-molt lethargus	75 hr	56 hr	39 hr
Egg-laying begins	~90 hr	~65 hr	~47 hr
Egg-laying maximal	~140 hr	~96 hr	~62 hr
Egg-laying ends	~180 hr	~128 hr	~88 hr
Length at first molt	360 µm	370 µm	380 µm
Length at second molt	490 µm	480 µm	510 µm
Length at third molt	650 µm	640 µm	620 µm
Length at fourth molt	900 µm	850 µm	940 µm
Length at egg-laying onset	1150 µm	1060 µm	1110 µm
Maximal egg-laying rate	5.4/hr	9.1/hr	8.1/hr
Total eggs laid	275	280	170

Figure 1.1: Jean Nicot presenting the tobacco plant to Queen Catherine de Medicis and the Grand Prior of the House of Lorraine 1655 [i.e., 1561] (Bros, 1868).

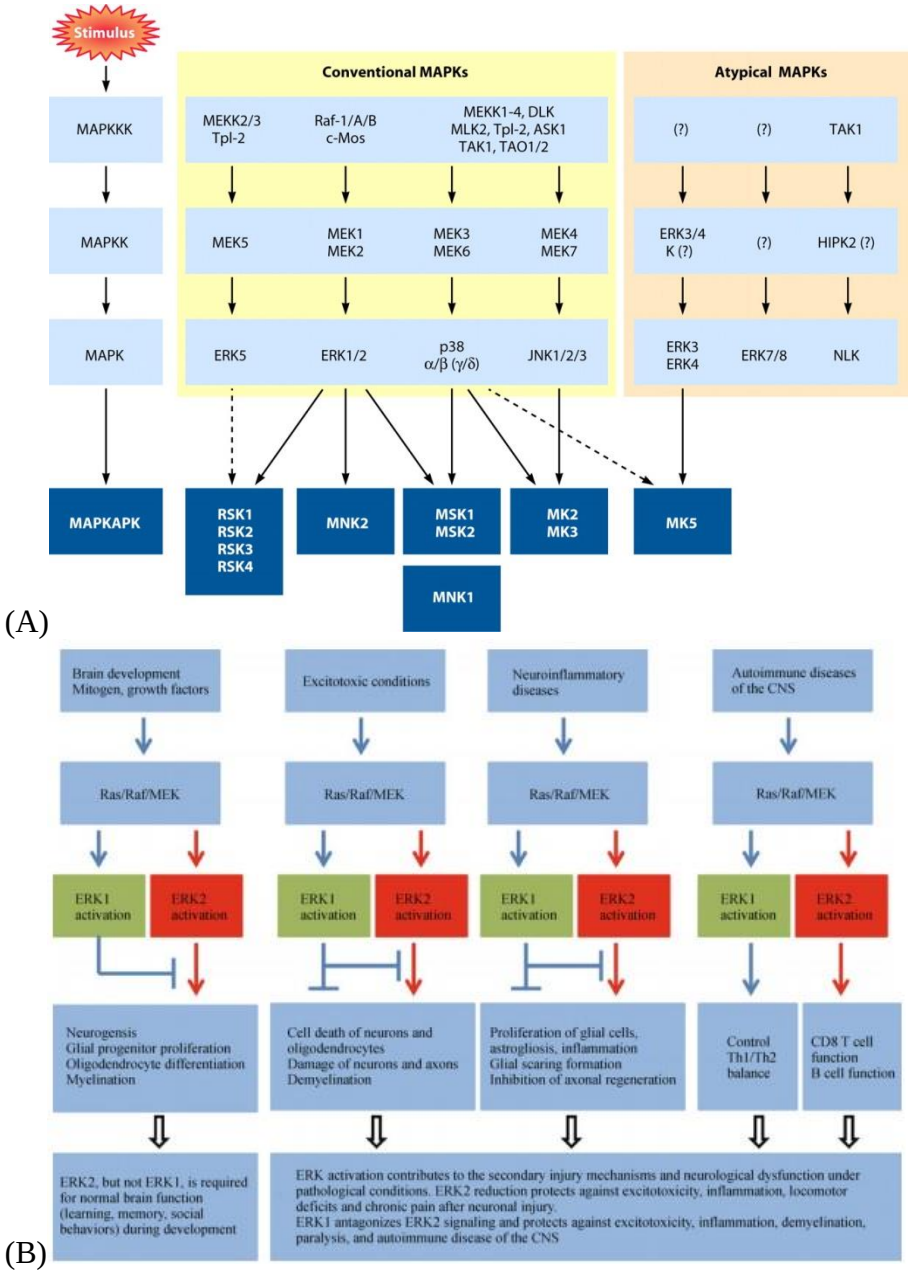


Figure 1.2: (A) MAPK signaling cascades (Cargnello & Roux, 2011). (B) Functions of ERK isoforms in the pathophysiology of the central nervous system (Yu, 2012).

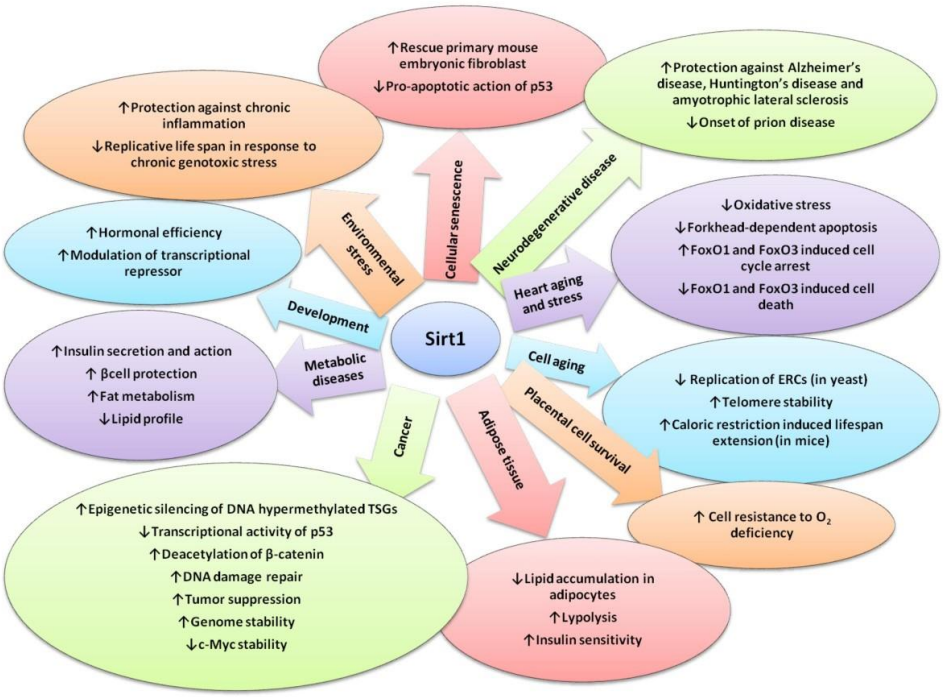


Figure 1.3: Biological functions of sirtuin SIRT1 (Rahman & Islam, 2011).

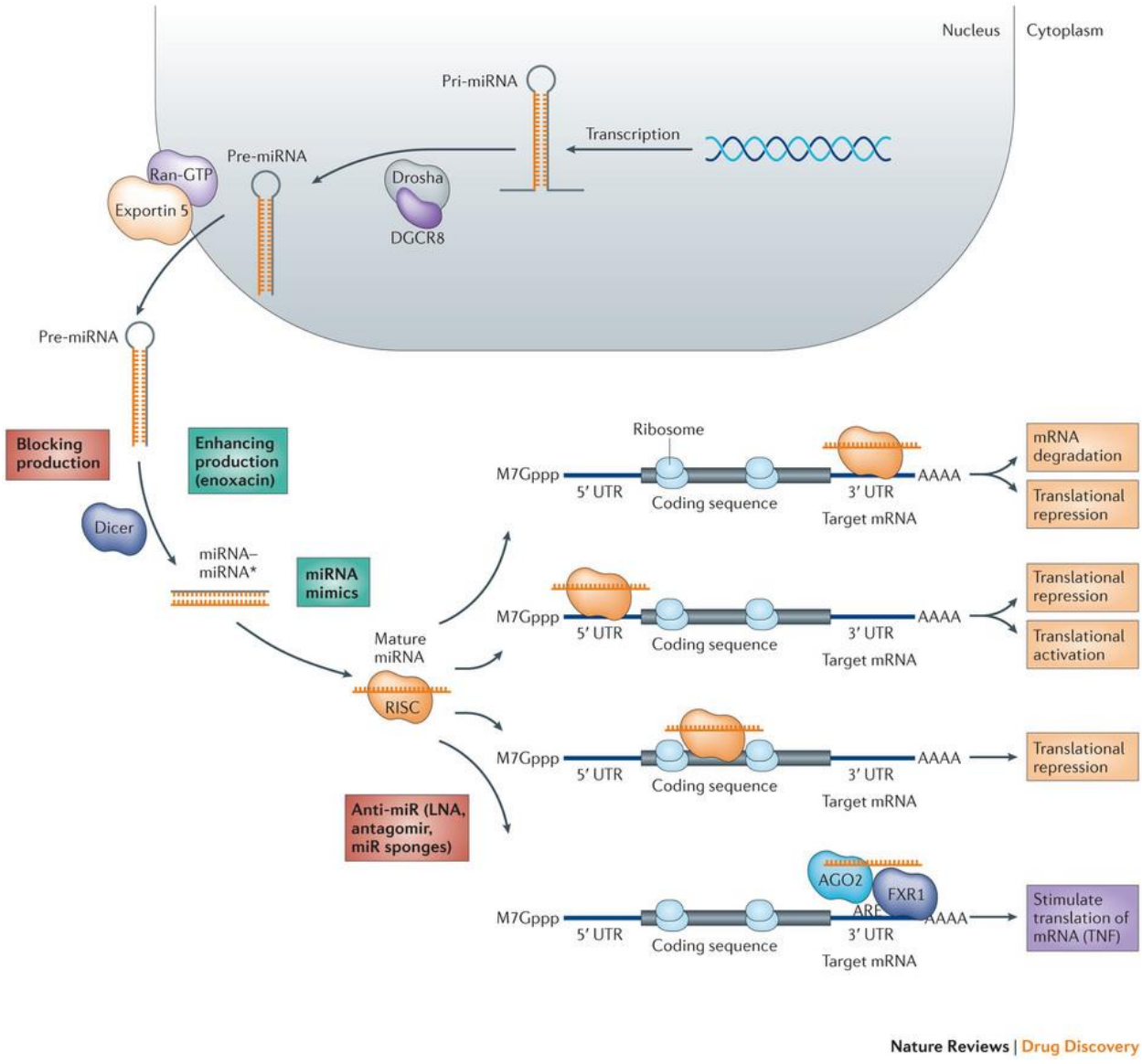


Figure 1.4: MicroRNA biogenesis, regulation, and function (Ling, Fabbri, & Calin, 2013).

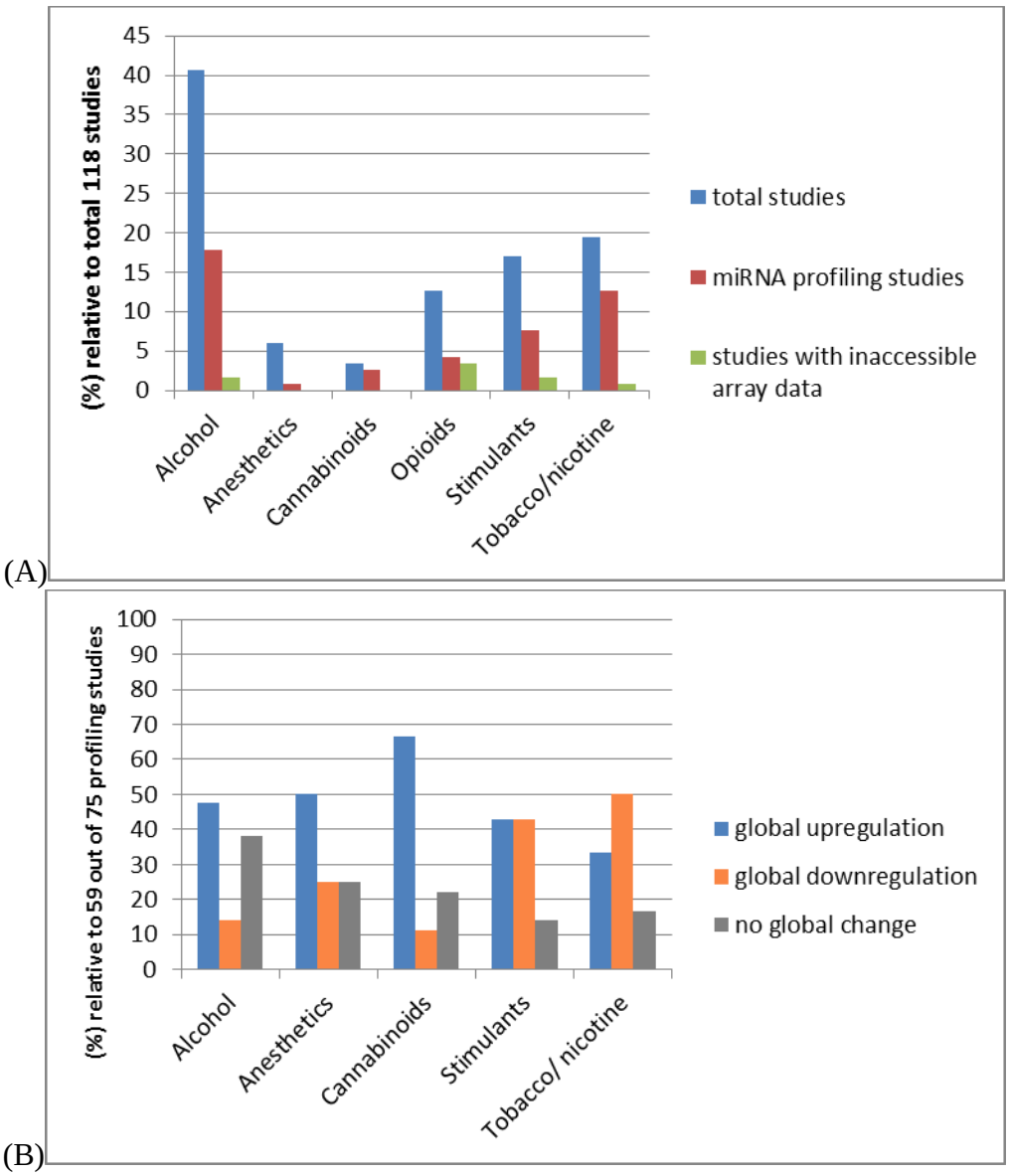
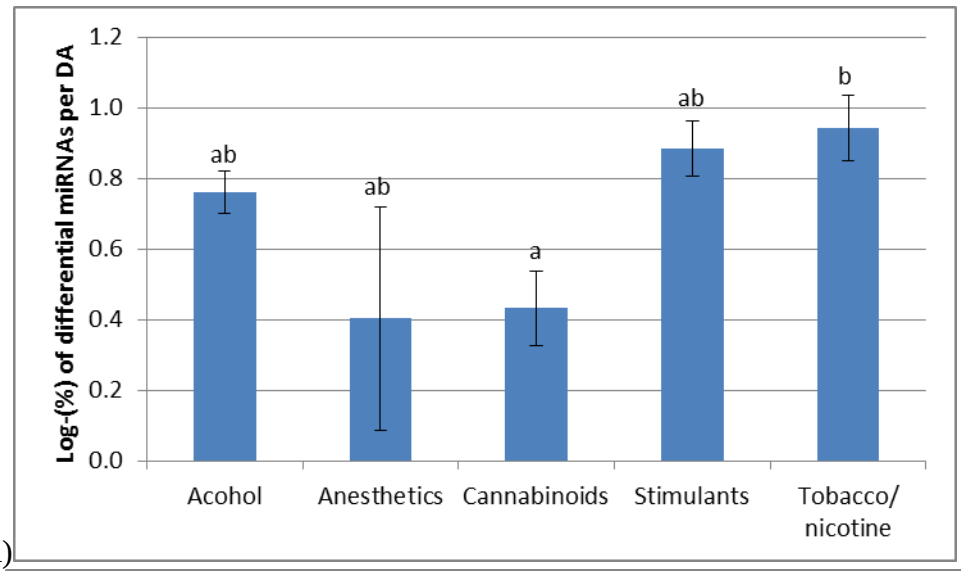


Figure 1.5: (A) Distribution of miRNA studies among DA (Drugs of Abuse). (B) Frequencies of studies reporting directional shifts in global miRNA expression profiles (F. A. Taki et al., 2015).

(A)



(B)

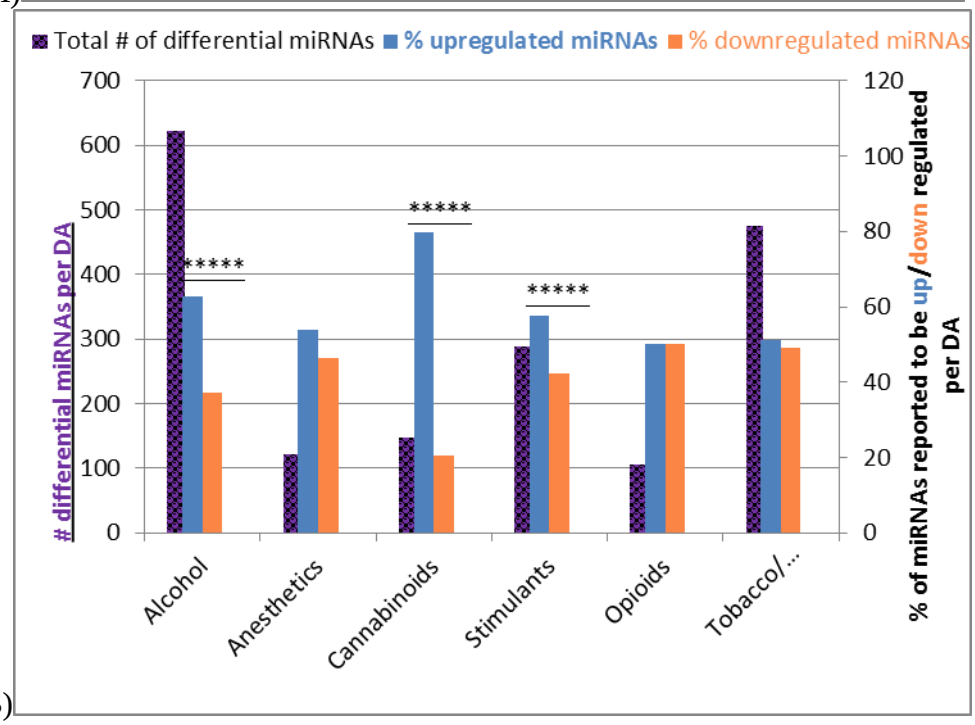


Figure 1.6: (A) The % of differential miRNAs as a function of DA from 54 global profiling studies (73 different treatment conditions). Note that Opioids had only 1 profiling study and therefore was not included in the analysis. ANOVA followed by post-Hoc pairwise comparisons was performed with $p<0.05$. Different letters denote statistically significant differences. (B) Total number (#) of miRNAs reported to be differential (Left axis) and the (%) of miRNAs reported to be up or down-regulated (right axis) in response to a DA in all 118 studies. Chi square test was used to compare the difference between induced vs. suppressed miRNAs per DA ($p<0.05$) (F. A. Taki et al., 2015).

Chapter II

Tri-generational microRNA profile shifts in response to parental post-embryonic nicotine exposure

Abstract

Early developmental stages are highly sensitive to stress and it has been reported that pre-conditioning with tobacco smoking during adolescence predisposes those youngsters to become smokers as adults. However, the molecular mechanisms of nicotine-induced transgenerational consequences are unknown. In this study, we genome-widely investigated the impact of nicotine exposure on small regulatory microRNAs (miRNAs) and its implication on health disorders at a transgenerational level. Our results demonstrated that nicotine exposure, even at the low dose, affected the global expression profiles of miRNAs not only in the treated worms (F0 parent generation) but also in two subsequent generations (F1 and F2, children and grandchildren). Some miRNAs were commonly affected by nicotine across two or more generations while others were specific to one. The general miRNA patterns followed a "two-hit" model as a function of nicotine exposure and abstinence. Target prediction and pathway enrichment analyses showed *daf-4*, *daf-1*, *fos-1*, *cmk-1*, and *unc-30* to be potential effectors of nicotine addiction. These genes are involved in physiological states and phenotypes that were consistent with previously published nicotine induced behavior. Our study offered new insights and further awareness on the transgenerational effects of nicotine exposed during the vulnerable post-embryonic stages, and identified new biomarkers for nicotine addiction.

Key words: nicotine, systemic miRNA profiles, *C. elegans*, post-embryonic stage, withdrawal, transgenerational impact

Introduction

40% of children have been estimated to be exposed to nicotine and up to 60% of teenagers were reported to be active smokers in some developing countries in 2010 (WHO, 2012). Whether actively or passively taken, tobacco smoke causes damage to every organ in the body. High death rates and increased economic burden have triggered a lot of policies and research on tobacco-related diseases. Despite its known health risks, tobacco abusers are entrapped in a vicious cycle of drug dependence. Among the 4000 chemicals in tobacco, nicotine is one of the primarily addictive components mediating continuous smoking relapses throughout an individual's lifetime (CDC, 2010a; USDHHS, 1988). Early developmental periods are known to be highly sensitive and vulnerable to stress. Children who are exposed to smoke are more susceptible to become smokers during adulthood (Kandel & Chen, 2000; Slotkin, 2002). Numerous studies have reported the effects of nicotine (Neal L. Benowitz, 1988; Counotte et al., 2009; Dani & Heinemann, 1996; Feng et al., 2006; Samaha, Yau, Yang, & Robinson, 2005; Sobkowiak, Kowalski, & Lesicki, 2011; Thomas, Welsh, Galvez, & Svoboda, 2009; Wada et al., 1989) which focused on the exposed generation. Other studies investigated the effects of nicotine during prenatal and perinatal periods (Ajarem & Ahmad, 1998; El Marroun et al., 2014; Holloway, Cuu, Morrison, Gerstein, & Tarnopolsky, 2007; Maritz, 2013). However, to our knowledge, the transgenerational effect of nicotine exposed strictly till adolescence has not been reported. Our recent studies show that nicotine exposure caused significant behavioral changes in *C. elegans* (F. Taki et al., 2013). However, the molecular mechanisms of nicotine-induced transgenerational consequences are not known. Therefore, elucidating the molecular regulatory mechanism for such phenotypes is essential for developing new approaches for monitoring and treating nicotine-related health problems.

Research on nicotine was based on many biological organisms (rats, mice, flies, fishes, and worms) (Matta et al., 2007) that modeled different aspects of nicotine-induced behavior like sensitization, tolerance, withdrawal, and reinforcement. We employed the nematode *C. elegans* to investigate the mechanism of action of nicotine on multiple levels. Research on *C. elegans* is relatively inexpensive, practical, and free of ethical concern. *C. elegans* is particularly attractive for transgenerational studies due to its small size (1 mm adult), short generation time (2-3 days) and lifespan (2-3 weeks) at 20^{o}C, and superfluous offspring production (300 eggs/N2 hermaphrodite) (Riddle et al.). *C. elegans* shares up to 80% homology with the human genome (Beitel, Clark, & Horvitz, 1990; S. Brenner, 1974). Thus, it has provided a wealth of information about biological and physiological processes over the last decade, the most recent of which involved the discovery of the microRNAs (miRNAs) (R. C. Lee et al., 1993).

Serious research has been devoted to dissect the factors involved in gene regulation and has provided clues concerned with the environmental contribution in shaping physiological phenotypes. miRNAs are an extensive class of newly discovered small regulatory RNAs (B. Zhang et al., 2007). Over 200 and 1000 miRNAs have been sequenced in *C. elegans* and humans, respectively. Due to their conserved and pleiotropic roles in gene regulation processes (Ambros, 2003; Aukerman & Sakai, 2003; C. Z. Chen, Li, Lodish, & Bartel, 2004; V. N. Kim, 2005; McManus, 2003), miRNAs are considered biomarkers of an innate response to environmental fluctuations. Nicotine altered miRNA expression levels in different biological systems (e.g. PDLSC, mouse fetal neuroepithelial precursors, rodents and PC12 cell model, canines, humans) (Balaraman, Winzer-Serhan, & Miranda, 2012; W. Huang & Li, 2009; Kassie et al., 2010; Ng et al., 2013; Shan et al., 2009; Shin et al., 2011). Some were linked to disruptions in stem cell regeneration (Ng et al., 2013). Others were involved in tumorigenesis (e.g. let-7,

miR-16 and miR-21) (Shin et al., 2011; Baohong Zhang, Pan, Cobb, & Anderson, 2007). Nicotine also antagonized and upregulated ethanol-induced miRNAs (Balaraman et al., 2012). Interestingly, a study demonstrated the role of miR-140* in nicotine addiction using rodents and PC12 cells (W. Huang & Li, 2009). The research showed that miR-140* targeted dynamin, which is crucial for neuronal plasticity and hence addiction-related processes (W. Huang & Li, 2009). Taken collectively, these studies showed a role for miRNAs in nicotine-dependent mechanisms. In this study, we systematically investigated the transgenerational impact of nicotine on miRNA expression and its implication on nicotine-induced health disorders. We took advantage on the wealth of data available on nicotine dose response curves. The latter are specific to each model organism and experimental design, yet are phenotypically comparable. Thus, we chose two nicotine doses associated with a stimulatory versus depressive effect on speed in our *C. elegans* model as reported by Sobkowiak and colleagues (Sobkowiak et al., 2011).

Material and Methods

Nicotine exposure and sampling

Nicotine exposure and worm sampling were based on our previous method (F. Taki et al., 2013). Briefly, nicotine (Acros Organics, NJ, USA) was dissolved in phosphate buffer as 1 M and 0.001 M stocks. NaCl, peptone, agar and water mixture were first autoclaved, cooled and then kept at 55 °C. After the addition of cholesterol, $CaCl_2$, $MgSO_4$ and KPO_4, the medium was divided into three flasks. The first flask was dedicated for control plates (no nicotine) and was left as is. Conversely, an equal amount of nicotine solution was added from each stock to the respective flask to give final concentrations of 20µM and 20mM in the medium, respectively.

Thus, worms were exposed to nicotine uniformly distributed in solid agar medium. The selection of nicotine concentrations (20µM and 20mM) was based on previous reports (Matta et al., 2007; Sobkowiak et al., 2011) followed by a set of assays to confirm the dose-dependent biphasic phenotypes. Subsequently, we performed extensive analyses on the effect of nicotine on sinusoidal movement, body bends and reversals, as well as speed (F. Taki et al., 2013). The chosen concentrations were associated with a non-monotonic bell shaped response and thus agree with previous reports. So, 20µM and 20mM nicotine were considered suitable for our current and intended miRNA study.

C. elegans hermaphrodite N2 Bristol wild type was used. Maintenance and worm transfer were done after NGM plates were seeded with OP50, and then kept at 20^{o}C. Egg synchronization was done via bleaching method (J. Sulston & Hodgkin, 1988) with slight modifications. Bleach breaks down the worms allowing for eggs to be free in solution. The eggs were then washed several times and were left to be suspended in the last wash on a shaker in the 20^{o}C incubator for about 14 hours. After hatching, all progeny were stuck at L1.

L1 larvae of the parent (F0) generation were transferred to the three treatment groups which included the control group along with the low and high nicotine concentrations. Parent (F0) exposure lasted for about 30 hours until L3/L4 transition. Then, worms were washed off the plates into two eppendorf tubes and pelleted. We controlled for sample bias by collecting all worms on a plate. Thus each biological replicate included worms with variable sensitivities and covered the entire response spectrum within a plate. The bigger pellet was intermittently centrifuged two times at 2000 rpm then 3000 rpm with supernatant removal. Then, it was flash frozen in liquid nitrogen, and stored at -80^{o}C for subsequent molecular studies. The eppendorf with the smaller pellet was washed twice with M9 interrupted by centrifugation and supernatant

removal. The worms were then transferred to nicotine-free NGM plates seeded with OP50, left to dry, and were then sealed and placed back in the 20^{o}C incubator to grow until second day of adulthood (egg laying peak). Worms were then collected for synchronization of isolated eggs. The whole procedure was repeated twice until F2 generation.

miRNA expression profile

Total RNA was extracted for all treated and control samples using mirVana™ miRNA Isolation Kit. Briefly, the sample was denatured using a lysis buffer. RNA was then separated from DNA and proteins via acid-phenol extraction. Then, ethanol was added to the sample followed by centrifugation to allow it to pass through a glass-filter. Several washes preceded the elution of the RNA with DNase/RNase-free water. RNA quantification and evaluation was done using the NanoDrop ND-1000 Micro-Volume UV/Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Reverse transcription was performed using TaqMan microRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) to reverse transcribe extracted RNA to cDNA for all 231 miRNAs . A total of 200ng of RNAs was used for each RT reaction. The reactions were then run using thermal cycler for 16^{o}C for 30 min followed by 42^{o}C for 30 min, 85^{o}C for 5 min and finally held at 4^{o}C. The cDNAs were then diluted in 80µl DNase/RNase-free water for qRT-PCR.

The expression levels of miRNAs were analyzed after performing qRT-PCR on 384-well-plates using the ViiA^{TM} Real-Time PCR System (Applied Biosystem). Briefly, each well carried a 15µl reaction of 5.5µl DNase/RNase free water, 7.5µl SYBR Green master mix, µl diluted cDNA, 1µl primer mix. A minimum of 3 biological replicates were run. The reaction was

run for 10 min at 95^{o}C for enzyme activation followed by denaturation for 15 sec at 95^{o}C and an annealing/extension step for 60 sec at 60^{o}C. The latter 2 steps were repeated for 40 cycles.

The Ct values from the qRT-PCR were exported to an Excel file. The average of the total miRNA (231) Ct-values was used for normalization. The ΔCt values were calculated as $Ct_{(miRNA)}$ -$Ct_{(avg\ miRNAs)}$. The ΔΔCt was calculated as the difference in the ΔCt values between control and treatment. Then the fold change was calculated as $2^{(\Delta\Delta CT)}$. Statistical analysis was based on t-test for independent samples via SPSS (20) to compare each of the nicotine treatments with control (i.e. low vs. control and high vs. control). Welch test correction was performed to account for unequal variance in each of the compared groups (control and treatment). In addition, to increase the statistical stringency, miRNAs differential expression was deemed significant only if it fulfilled two criteria: p value <0.05 and expression changed by at least 50% relative to control.

Fold change values ($2^{(\Delta\Delta CT)}$ -1) were used to construct heat maps coupled with non-supervised hierarchical clustering using Euclidean distance and complete linkage analysis and included all miRNAs (vertical axis) and samples (six treatment groups on the horizontal axis). The latter approach was done for miRNAs with statistically significant expression alterations using MeV (MultiExperiment Viewer) (Saeed et al., 2006).

Target prediction and pathway analysis

Differentially altered miRNAs that were common to at least two generations were used to perform target prediction using mirSOM software (Heikkinen, Kolehmainen, & Wong, 2011). To prepare the input for analysis, duplicates were removed and thus only unique values of targets with a perfect seed match were used. The predicted targets were ranked according to the frequency of occurrence in the originally compiled gene list. Such a frequency reflects the number of miRNAs predicted to target a gene. The list was used as input for DAVID (d. W.

Huang, Sherman, & Lempicki, 2009a, 2009b) for analysis. Gene ranking was based on functional annotation clustering (highest stringency) endpoint provided by DAVID. Target genes belonging to clusters with enrichment values ≥2 were used based on the order of the clusters to prepare a ranked list. The latter included 321 genes and was used as an input for GOrilla (process ontology) (Eden, Navon, Steinfeld, Lipson, & Yakhini, 2009) that provided DAG (directed acyclic graph) showing relationships among enriched processes. miRNA-target networks were constructed using Cytoscape (Smoot, Ono, Ruscheinski, Wang, & Ideker, 2011). Other specific analysis were performed and described in details in their corresponding result section.

Results

Analyzing the intra-generational effect of nicotine

The effect of nicotine on the parent (F0) generation

We studied the effect of nicotine on the expression levels of 231 miRNAs in L4 *C. elegans* (N2). The effect of nicotine on the F0 generation can be found in our previous study (F. A. Taki et al., 2013). To summarize the results, nicotine affected the expression of forty miRNAs (17.3%), in which three miRNAs (miR-79, miR-80 and miR-230*) were altered in response to the lower nicotine concentration (20µM). The expression of 37 miRNAs was changed after high nicotine concentration (20mM) treatment. About 78% of the altered miRNAs were upregulated with fold changes ranging from 0.5 to 3.0 folds. Twenty six miRNAs were upregulated by >0.5 folds ($p<0.05$). The remaining 22% miRNAs were downregulated by the 20mM nicotine treatment. miR-80 was the only miRNA consistently upregulated in worms treated with low or high nicotine concentrations. Based on the miRNA profile changes observed in response to nicotine in the F0 worm population, we decided to continue our investigations to explore the

indirect transgenerational effect of nicotine treatment limited to the postembryonic-stages of the F0 generation.

The effect of nicotine on the offspring (F1) generation

The F0 generation was the only one with direct contact with nicotine, and such was limited to the 30-hour, post-embryonic period (i.e. L1 to L4). With this in mind, we investigated possible effects of nicotine on the genome-wide miRNA expression levels in L4 belonging to the F1 generation. As shown in Figure 2.1A, the expression of thirty one (13.4%) unique miRNAs was altered in response to parental nicotine exposure. The low and high nicotine concentrations affected nine and twenty six miRNAs, respectively. Three out of nine miRNAs were upregulated in offspring of worms exposed to the lower nicotine concentration (20µM) (Table 2.1). The highest increase was observed for miR-254 (3.5, p=0.006) followed by miR-793 (1.2, p=0.03) and miR-66 (0.7, p=0.04). On the other hand, the remaining six miRNAs were downregulated with the highest fold change observed for miR-253 (-1.0, p<0.001) followed by miR-255 (-0.7, p=0.001), miR-786 (-0.6, p=0.002), and miR-785, miR-794, and miR-41 (-0.5, p=0.036, p=0.029, and p<0.001, respectively) (Table 2.1). As for offspring of parents exposed to the higher nicotine concentration (20mM), 32% of the differentially altered miRNAs were upregulated. The highest upregulation was observed for miR-254 (6.4, p=0.018) followed by miR-260 (5.6, p=0.023), miR-257 (4.8, p=0.034), miR-36 (2.9, p=0.014), miR-1019 (1.6, p=0.005), miR-236 (0.8, p=0.01), miR-353 (0.7, p=0.016), and miR-262 (0.6, p=0.014). Conversely, 68% of the miRNAs, altered after parental exposure to high nicotine concentration, were downregulated. With the highest fold change being for miR-253 (-1.0, p<0.001), others like miR-1829a (p=0.006) , miR-1829b (p=0.001),miR-2208a* (p=0.041), miR-56* (p=0.005), miR-71 (p=0.011), miR-1 (p=0.02), miR-85 (p=0.01), miR-1829c (p=0.027), miR-785

(p=0.002), miR-53 (p=0.001), miR-2212St (p=0.037), miR-2218a (p=0.044), miR-42 (p=0.013), miR-1828 (p=0.025), miR-242 (p=0.024), and miR-784* (p=0.022) decreased by more than 0.7 folds (Table 2.1). Additionally, we observed that three miRNAs were affected by both nicotine concentrations when exposed during the parental post-embryonic stage. miR-254 was the most upregulated in offspring of both treatment groups and as the concentration increased, the magnitude of the upregulation was more dramatic as it doubled from 3.0 folds to 6.0 folds. On the other hand, miR-785 (-0.5) and miR-253 (-1.0) were downregulated in response to both nicotine concentrations exposed to parents, but unlike miR-254, the magnitude was similar in offspring of parents exposed to both low and high nicotine concentrations (Table 2.1).

The effect of nicotine on the grand-offspring (F2) generation

We traced down the effect of nicotine until the F2 generation. Similarly, grand-offspring were never exposed to nicotine, but progressed from ancestors treated with nicotine during their post-embryonic period. In F2, sixteen unique miRNAs (6.9%) showed statistically significant alterations in their expression levels in response to grand-parental nicotine exposure (Table 2.2). Ten and twelve miRNAs were differentially expressed in grand-offsping of parents exposed to low and high nicotine concentrations, respectively. Among the affected miRNAs, 40:60% and 50:50% were down and upregulated in grand-offspring originating from the parental low and high nicotine treatment groups, respectively (Table 2.2). Interestingly, six of the sixteen unique miRNAs were commonly and similarly altered in both treatment groups progressing from grand-parents exposed to low and high nicotine concentrations. miR-80 was the only commonly upregulated miRNA by 0.8 (p=0.027) and 0.6 (p=0.035) fold changes in grand-progenies of parents exposed to low and high doses, respectively. Oppositely, miR-239a ($p_{(L)}$=0.006, $p_{(H)}$<0.001), miR-240 ($p_{(L)}$=0.012, $p_{(H)}$=0.001), and miR-232 ($p_{(L)}$<0.001, $p_{(H)}$=0.015) were

downregulated in both groups by about 0.6 folds, while miR-53 and miR-244 were downregulated by about 1.0 folds with $p_{(L)}$ and $p_{(H)}$ <0.001. Other miRNAs were dose-dependent as their expression levels changed in one of the two groups, but not both. For example, miR-235 (p=0.003), miR-51 (p=0.043), and miR-790 (p=0.041) were upregulated by >0.5 folds, while miR-255 was downregulated by about 0.5 folds (p=0.009) in grand-offspring of 20µM-treated parents. On the other hand, the highest upregulation was observed for miR-1 (2.0, p=0.005) followed by miR-2218a (0.9, p=0.013), miR-2220 (0.8, p=0.001) and miR-800 and miR-2212* (0.5, p=0.049 and p=0.001, respectively) leaving miR-1828 to be uniquely downregulated by 0.5 folds (p=0.046) in grand-offpring of parents treated with high nicotine dose (20mM) (Figure 2.1B; Table 2.2).

Investigating the inter-generational effect of nicotine

Fourteen miRNAs were affected in more than one generation (Figure 2.1C)

We checked for miRNAs whose expression was altered in more than one generation and found that indeed some miRNAs were commonly affected. Four miRNAs were altered in both F0 and F1 worm populations in response to direct and indirect nicotine treatment, respectively. As shown in Figure 2.2, miR-242 and miR-1829b were upregulated (1.0 fold) in F0 worms exposed to high nicotine concentration, while both were downregulated (-0.5 folds) in their offspring. miR-794 was downregulated (0.5 folds) in F1 originating from the low nicotine treatment group without being affected in the parents, while it was upregulated (0.6) in the parents (F0) exposed to high nicotine concentration. Though the F0 parents exposed to 20µM nicotine were not associated with an alteration in miR-785, it was downregulated (-0.5) in their

F1 offspring. On the other hand, it reversed in response to the 20mM concentration as it was upregulated in F0 (1.0 fold) to become downregulated in their offspring by 0.5 folds.

The parents and the grand-offspring also shared four altered miRNAs in response to nicotine. Both miR-2220 and miR-800 were altered in both F0 parents treated with the high nicotine dose and their grand-offspring. The extent seemed to decrease from 3.4 to 0.8 folds in the case of miR-2220, while it remained similar (0.5-0.6 folds) for miR-800. miR-80 on the other hand was upregulated (1.0 and 0.9 folds) in both F0 parents exposed to low and high nicotine concentrations and remained like so (0.8 and 0.6 folds) in their grand-offspring, respectively.

The F1 offspring and F2 grand-offspring were grown in nicotine-free environments. Our results showed that out of the total miRNAs differentially expressed across all generations, five were common to F1 and F2. miR-53 and miR-1828 were downregulated (-0.5) in the F1 offspring of parents exposed high nicotine treatment group and continued to be so in the F2 grand-offspring (-1.0 and -0.5, respectively). Opposite patterns were observed for miR-2212* and miR-1 which started as being downregulated in F1 20mM treatment group to become upregulated in F2. The expression of miR-1, miR-2218s and miR-2212* was increased by almost 2.0 and 1.0 folds, respectively from F1 to F2generations, both of which originated from parents and grandparents exposed to the high nicotine concentration. No altered miRNAs were shared between F1 and F2 worms from parents and grandparents exposed to the low nicotine concentration.

Interestingly, the expression of only one miRNA was computed to be statistically significant across all generations. It appears that miR-255 was upregulated by more than 1.0 fold in F0 parents exposed to the high nicotine concentration. Neither this pattern, nor its inverse was observed in the succeeding generations. However, our data shows the miR-255 was

downregulated (-0.7 and -0.5 folds) in both F1 and F2 generations of parents and grandparents exposed to the lower nicotine concentration during its postembryonic development. Taken collectively, we report that fourteen miRNAs (6%) were differentially altered in response to nicotine in at least two generations.

Seventy two miRNAs were differentially regulated across all generations

We investigated the patterns of miRNA expression levels across all generations in response to post-embryonic nicotine exposure in *C. elegans*. We compiled a list of all miRNAs whose expression was altered by at least 0.5 folds ($p<0.05$) in at least one generation. As a result, the list included seventy two miRNAs which represent 31% of all miRNAs tested. We constructed a heat map based on the Mean Fold Change (MFC) values for both 20µM (Low) and 20mM (High) nicotine treatment group across all generations (Figure 2.3). Different columns represent MFCs of six groups and are relative to control (0µM nicotine). Then, unsupervised hierarchical clustering based on Euclidean distance and complete linkage was performed and included six groups. For optimal visualization of the patterns, leaf ordering was done for both miRNAs and samples. As shown on the map, expression patterns for treatment groups belonging to the same generation were mostly similar to one another when compared to other generations. Thus, they were closely ordered next to one another. Also, we noticed another general pattern where worms exposed to the 20mM (high) nicotine treatment group as well as their F1 progeny had more altered miRNAs than those belonging to the lower nicotine concentration. Such was evident in the colored cells relative to those that were black (i.e. red: upregulated, green: downregulated, black: no change). However, the situation was different in the F2 generation where grand-offspring worms from parents exposed to high and low nicotine doses had similar expression patterns. Interestingly, our data showed that worms exposed to the lower nicotine

concentration in F0 parents clustered with both F2 grand-offspring treatment groups. On the other hand, both groups in F1 had a different overall miRNA expression pattern and thus clustered separately from F0 and F2.

The fold changes of the seventy two miRNAs were also subjected to unsupervised optimized clustering. With 8.6 as a cutoff distance, results showed two clusters, one that included four miRNAs (miR-254, miR-260, miR-257, and miR-36), while the other included sixty eight. Decreasing the cut-off distance by half to 4.3 resulted in one additional cluster of five miRNAs (miR-2220, miR-66, miR-90, miR-2216*, and miR-2218b*).

For a more qualitative assessment of the expression changes across the three generations, we performed further analysis on the seventy two miRNAs that showed differential alteration in at least one generation. The exact values for the expression levels have been thoroughly discussed above. Hence, we provided further elaboration to study the effect of nicotine in a dose and generation-dependent manner. As shown in the radar graphs (Figure 2.4), each concentric ring represents respective fold changes with zero as no change. miRNAs were ordered alphabetically to allow for comparison between low and high treatment groups across generations. In F0, the direct nicotine effect was more dramatic in the high versus the low concentration treatment. As an overall, the pattern seen in response to the 20µM nicotine concentration was circular and generally uniform, bordering the zero-fold-rim, and did not exceed 1.0 fold change in either direction. On the other hand, the circular pattern was no longer uniform in worms treated with the 20mM nicotine dose. Much more miRNAs were increased by >1.0 folds, and the pattern borders mostly intersected with 1.0 fold-rim. In F1, though nicotine was no longer in direct contact, the progeny of worms exposed to the low nicotine concentration had more altered miRNAs than their parents. The pattern is no longer circular and was reformed

by dramatic up and downregulations that reached the 4th rim in the positive direction, and the 1st rim in the negative direction, respectively. A more defined shape was observed in the offspring of parents exposed to the high nicotine concentration as it had a star-shaped pattern in the F1 generation. The increase in the downregulated miRNAs was similar to offspring of the low nicotine concentration. However, the main shifts in expression levels were the doubled upregulations in three out of four miRNAs (miR-260, miR-254, miR-36, and miR-257). Finally in F2, the pattern went back to become circular. Nonetheless, unlike the F0 generation, the circular pattern was similar in grand-offspring originating from grandparents treated with either nicotine doses. The circular pattern generally overlapped with the F0 20µM treatment group, and this was consistent with results shown in the heat map after hierarchical clustering.

Investigating the nicotine potential effectors downstream of the altered miRNAs

Most miRNAs have pleiotropic functions. It is known that one gene can be targeted by up to hundreds of miRNAs. Vice versa, one miRNA can target hundreds of genes. Therefore, to understand processes that are mostly affected by nicotine treatment, we limited our analyses on the fourteen miRNAs that showed differential expression in at least two generations (Figure 2.2). Thus, a list of genes predicted to be targeted by miR-242, miR-1829b, miR-785, miR-1, miR-2218a, miR-53, miR-235, miR-80, miR-2220, miR-800, miR-255, miR-1828, miR-2212*, and miR-794 was compiled. The list included 2462 unique gene suspects. Two genes (*rbc-1* and *egl-10*) were predicted to be targeted by five miRNAs. Based on annotations from wormbase (Yook et al., 2012), *rbc-1* might be involved in proton-pump translocation, while *egl-10* has a role in egg laying and regulation of certain signaling pathways. Four miRNAs were predicted to regulate each of *F10D2.10*, *miz-1*(zinc-finger transcription factor), *cyp-23A1* (xenobiotic metabolism), *C50H11.8*, and *tag-97.44* genes were predicted to be targeted by three miRNAs.

348 were expected to be common to two miRNAs included in our analyses. The remaining genes were not considered as common targets to the fourteen miRNAs included in the target prediction analysis.

To investigate whether each miRNA is involved in particular pathways, a gene list for each of the fourteen miRNAs was used as an input for DAVID analysis. Based on the percentage of genes belonging to a certain process, an enrichment value is computed coupled with p-values (Fold enrichment, p-value). Enrichments manifested in KEGG pathways were associated with only four miRNAs. miR-1828 might have a role in the Wnt-signaling pathway (3.3, p=0.058). miR-80 was predicted to target genes involved in Notch (6.0, p=0.025), MAPK (3.0, p=0.049), ErbB (4.0, p=0.082) signaling pathways. Also, MAPK (2.4, p=0.095) as well as endocytosis (3.1, p=0.006), and pyrimidine metabolism (2.9, p=0.016) might be regulated by miR-785. Finally, miR-1 was estimated to target signaling pathways such as the phosphatidylinositol system (5.7, p=0.003), lysosome (2.8, p=0.02), oxidative phosphorylation (2.2, p=0.027), Wnt (2.7, p=0.035), inositol phosphate metabolism (5.24, p=0.036), and mTOR signaling pathways (3.9, p=0.075).

Since the miRNA annotation research is still in the juvenile stages, we were interested in investigating possible relationships between all the genes predicted to be targeted by the fourteen miRNAs. In order to do that, we used GOrilla software which provides a DAG (Directed Acyclic graph) that shows relationships among enriched pathways predicted to be regulated. The input for GOrilla was ranked. To prepare the ranked list, we performed functional annotation clustering for the 2462 genes through DAVID with highest stringency classification. The output from DAVID included 120 clusters arranged in decreasing order of enrichment values. Genes belonging to clusters with enrichment values greater than two were considered and were ranked in decreasing order (from most to least enriched). The latter list was composed of 141 ranked

genes and was used for input in GOrilla. The highest enrichments belonged to positive regulation of translation (10.9, $p=10^{-3}$) followed by macromolecule modifications (6.67-10, $p<10^{-3}$) (e.g. peptidyl-lysine and amino acid modification, protein metabolism). The fourteen miRNAs were computed to target metabolic and biosynthetic regulatory genes (4.6, $p<10^{-9}$). Transcriptional control as well as RNA, nitrogen and nucleobase metabolism were also highly enriched (4.6, $p<10^{-9}$). Processes like taxis and chemotaxis, axon guidance had an enrichment value of 2.9 with $p<10^{-7}$. Targeted processes like response to external ($p<10^{-5}$) and chemical stimuli ($p<10^{-3}$), cellular development ($p<10^{-3}$) as well as phosphorylation ($p<10^{-3}$) had enrichment values ranging from 2.1 to 2.7 (Figures 2.5 and 2.6A).

Then, a bottom-top approach was followed starting with matching genes of every enriched pathway to their predicted regulatory miRNAs. Based on GOrilla predictions (Figures 2.5 and 2.6AB), miR-242 was not involved in any of the enriched processes. miR-53 and miR-1829b targeted genes involved in molting cycle and locomotion. In addition to these two processes, miR-800 might regulate cellular development, response to stimulus. Interestingly, targets of miR-785 were restricted to nitrogen, nucleobase, and RNA metabolic processes, biosynthesis, and transcriptional regulation. On the other hand, miR-235 covered many of the enriched processes, except for taxis, and other functions related to protein metabolism and modifications. Six of the remaining miRNAs (i.e. miR-2218s, miR-794, miR-255, miR-2212*, and miR-80) might be working redundantly as they covered all processes except for those related to protein metabolism and some modifications. Finally, the last two miRNAs were the most pleiotropic and were anticipated to fine-tune genes encoding of the enriched functions except for cellular development and peptidyl lysine modification as was the case for miR-2220 and miR-1, respectively.

Pathways with the highest enrichment values and statistical significance

We narrowed our analysis further by focusing on select pathways predicted by GOrilla to be altered in response to nicotine (Figure 2.7A). Enrichment values >3.0 and p-values $<10^{-9}$ were used as cutoff criteria. Out of the fifty two overall pathways, only fourteen were then chosen for downstream analysis. 25 genes involved in those pathways were then extracted and used as input for gene functional annotation through DAVID. Such was run with highest stringency and resulted in two clusters. The first one, including *unc-30*, *skn-1*, *nhr-49*, *fos-1*, *nfi-1*, and *vab-3*, had an enrichment value of 21.4. The second one, including *daf-4*, *daf-1*, *cmk-1*, *pek-1*, and *sma-6*, had a lower enrichment value of 11.3. As detailed in Table 2.3, their functions include response to stress, development and differentiation, behavior such as egg laying and locomotion. These results were consistent with the overall target prediction discussed in the previous paragraphs.

Analysis through GOrilla provided a general idea about the enriched pathways across the three generations; however, it might have masked differences between individual generations. We wanted to investigate common effectors between only two generations. For F0-F1 pair, target prediction was done for five differentially altered miRNAs (miR-242, miR-785, miR-1829b, miR-794, and miR-255). For F0-F2 pair, also five miRNAs were used (miR-255, miR-2220, miR-80, miR-800, and miR-235). For F1-F2 pair, (miR-1, miR-2218a, miR-2212*, miR-1828, miR-53, and miR-255) were used for target prediction. The resulting gene lists included 1047, 1046, and 1149 genes for F0-F1, F0-F2, and F1-F2 pairs, respectively. The latter were individually run through DAVID for functional annotation clustering with highest stringency. Only clusters with enrichment values >3.0 were considered and the corresponding genes were extracted. Based on these criteria, F0-F1 pair didn't show any clusters. F0-F2 pair had one

enriched cluster (regulation of biosynthetic process, E=5.9) and included 16 genes. The F1-F2 pair had three enriched pathways involved in the regulation of biosynthesis and transcription (E_{avg}=4.2) and included a total of 15 genes. The absence of enriched pathways from target genes predicted to be regulated by miRNAs common to the parents and their offspring in the F0 and F1 generations suggests that different processes were altered in F0 versus F1 generations. This result is consistent with the hierarchical clustering where the distance between F0 and F1 was the highest, suggesting least commonalities in miRNA expression patterns.

unc-30, *fos-1*, *daf-4*, *daf-1*, *cmk-1*, *sma-1*, and *nfi-1* were common genes among the outputs from the above two approaches. Of the latter, only *unc-30*, *fos-1*, *daf-1*, *daf-4*, and *cmk-1* were targeted by differentially altered miRNAs in at least five of the six treatment groups across all generations. *daf-4* was predicted to be targeted by twenty two miRNAs, *daf-1* and *fos-1* were predicted to be targeted by fourteen and twelve miRNAs, respectively, while *cmk-1* and *unc-30* were targeted by eight and seven miRNAs, respectively (Figure 2.7A). Figure 2.7B depicts these five genes targeted by one or more of the fourteen commonly altered miRNAs (Figure 2.7; Table 2.3).

Discussion

Both genetics and environment determine the phenotype. Stress exposed during early development till adolescence has enduring effects (e.g. neuro-remodeling, sensitivity to drugs of abuse). A study showed that chronic nicotine exposure during adolescence but not adulthood lead to long-lasting alterations in the cognitive performance in rats (Counotte et al., 2009). Epigenetic modifications might be mediators of these short or long-term, and even transgenerational changes (Crews et al., 2012). To be considered as truly transgenerational, effects should remain evident in at least three or four generations depending on the ancestral

exposure period. Those that occur in impregnated individuals should leave a mark on four or more generations, while experiences limited to ancestral postnatal period or non-gravid adults impact three or more generations (Skinner, 2008). In our study, post-embryonic nicotine exposure was restricted to F0 ancestors prior to their sexual maturity. This exposure was associated with an altered miRNA expression profile that remained evident in three generations. Therefore, it modeled a true transgenerational effect.

Our results support the "two-hit" model proposed by Crews et al. (Crews et al., 2012). The first "hit" was the chronic nicotine exposure during the post-embryonic stage in *C. elegans*. The second "hit" was the withdrawal-associated stress experienced during gestation. Together, they caused alterations in the subsequent progeny (larvae) at the miRNA level, the focus of our study. Interestingly, some of the effects were shared while others were context dependent (e.g. nicotine dose, exposure vs. abstinence, acute vs. chronic, developmental period) (Figure 2.7C).

Concentration specific patterns

Chronic nicotine treatment was limited to about 30 hours from L1 to L4 in F0 generation worms. The subsequent generations were not in direct contact with nicotine. Despite that, nicotine affected miRNA profiles in a dose-dependent manner. In worms treated with the low nicotine concentration, the percentage of differentially expressed miRNAs remained similar across the generations (1-4% of miRNAs). However, a decreasing pattern was observed in worms treated with the high nicotine concentration as the generations progressed. 16.5% of the miRNAs had disparate expression in response to the direct exposure to the high nicotine concentration. The percentage decreased to about 11% in their F1 offspring. In the grand-offspring (F2), only 5.2% of the miRNAs showed changes in their expression patterns. This

implies that nicotine treated chronically and strictly during the adolescent stages of the parent generation had a long lasting effect that was still detected in the third generation. Moreover, the dose-dependent effect was also passed along the generations as the severity of withdrawal was proportional to the nicotine dose and duration of exposure (Skjei & Markou, 2003). Unlike the high nicotine concentration, the low nicotine dose was pharmacologically active but asymptomatic. Such a dose-dependent effect decreased but remained evident in each generation. The F0-initiated nicotine ripple faded as the generations progressed to reach a baseline adaptive response observed in worms succeeding from the 20μM (low) nicotine treatment group.

Generation versus nicotine concentrations

Some miRNA expression alterations were specific to one nicotine dose, but not the other, while the expression of other miRNAs was altered in response to both nicotine concentrations. The difference was mostly evident in the F0 generation where worms were in direct contact with nicotine. Only 2.5% were commonly affected in response to the low and high nicotine concentrations. However, the miRNA response almost tripled in F1 offspring as 9.7% miRNAs were commonly responsive to both low and high nicotine concentrations. The percentage continued to increase in F2 grand-offspring as 37.5% of the miRNAs were commonly affected in response to both high and low grandparental nicotine exposures.

Generation specific patterns

31.2% of the miRNAs were totally altered in response to both nicotine concentrations. As the generations progressed, the number of totally altered miRNAs decreased from 17.3% in F0 to 13.4% in F1 and finally became 6.9% in F2. Each generation had a pattern generally consistent between the two nicotine concentrations. About 78% of the miRNAs were upregulated in F0.

However, most of the miRNAs (67%) were downregulated in F1, while almost half of them (55%) were upregulated in the F2 worm population.

How can we explain the opposite miRNA patterns observed in F1 while both F0 and F2 were similar?

Nicotine exposure was limited to the F0 generation. Similar miRNA profiles were observed in F0 and F2, while F1 had an opposite pattern as shown in the heat map, and radar graphs. Revisiting our experimental settings, L4 larvae belonging to the F0 generation were chronically exposed to nicotine and thus were desensitized to it. The L4 larvae of the F1 generation were the progeny of F0 parents experiencing acute nicotine withdrawal. Finally, L4 larvae grand-offspring (F2) progressed from parents (F1) experiencing protracted withdrawal. This is depicted in Figure 7C where all three time points are elucidated by arrows.

It is crucial to recall two general nicotine-induced responses. The first is transient and results from either "acute" exposure or abstinence from nicotine. The second is adaptive and occurs after "chronic" treatment or abstinence from nicotine. Such might involve epigenetic modifications that stabilize gene expression in a dose and time-dependent manner in response to environmental changes. Thus, after adapting to a stimulus, an environmental shift back to "normality" is conceived as a new fluctuation. The system then responds transiently if the stimulus is temporary. Conversely, the response takes an adaptive, relatively permanent form in case the trigger is persistent. Thus, parent (F0) larvae model an adaptive state in response to chronic treatment. The perinatal period is vulnerable and sensitive to stress. It has been suggested that stress responses are transmitted through the germ line and are apparent in subsequent generations even in the absence of stressor (Crews et al., 2012). To further support the latter, *C.*

elegans maintains an epigenetic transcriptional memory through parental primordial germ cells (Furuhashi et al., 2010). Thus, grand-offspring (F2) larvae also model adaptation as they are the progeny of F1 parents experiencing chronic nicotine withdrawal. On the other hand, not only can chronic treatment followed by acute withdrawal disrupt a system, but contrasting symptoms can also arise after transitioning from acute to protracted withdrawal. With this in mind, the intermediate F1 generation does not model adaptation as their parents were experiencing acute withdrawal. Instead, they are sensitized and represent transience. This idea is clearly supported by an experiment done on rats where both direct nicotine exposure and chronic withdrawal were associated with a decrease in inhibitory control (i.e. addiction-related index). On the contrary, those rats undergoing acute withdrawal experienced improved inhibitory control (Kolokotroni, Rodgers, & Harrison, 2012). Collectively, the aforementioned scenarios can partly explain the similarities between F0 and F2 in contrast to F1 (Figure 2.7C).

Functional annotation of miRNAs whose expression was altered in response to nicotine

The miRNAs whose expression showed differential alterations in response to post-embryonic nicotine treatment in F0 belonged to many families. However, miR-51 and miR-80 families were more likely to mediate nicotine-induced effects. miR-51 family members are associated with a wide range of processes. Some of its members (i.e. miR-51, miR-52, and miR-54-56) might regulate the developmental timing by antagonizing let-7 and lin-4. This family was also hypothesized to regulate miRISC activity which increased after the loss of miR-51 members and others like miR-239a and miR-244. The latter two were among the miRNAs that showed differential expression across generations. miR-51 family members are broadly and highly expressed (Lim et al., 2003). Therefore further upregulation might hinder other miRNA activities by surpassing their miRISC complex binding (J. L. Brenner, Jasiewicz, Fahley, Kemp, & Abbott,

2010). Another abundant and constitutively expressed family is miR-58/80 family (Kato, de Lencastre, Pincus, & Slack, 2009; Lau, Lim, Weinstein, & Bartel, 2001; Lim et al., 2003). The deletion of all of miR-58 family members resulted in a sluggish worms with smaller body sizes. The miR-58 family members have a role in locomotion, body size and egg laying. Most of these traits are common with nicotine related phenotypes in *C. elegans* (e.g. paralysis in response to high nicotine concentration) (Matta et al., 2007). This might suggest a role of miR-58/80 family in nicotine molecular mechanism in the worm.

Eight major functional hubs were mapped through GOrilla. These included locomotion, response to stimulus, multicellular organismal process, single-organism process, developmental, cellular and metabolic processes, and biological regulation. The highest statistically significant enrichment values belonged to the biological regulation hub. The latter included the regulation of transcription and gene expression, metabolism (e.g. RNA, nucleobase-containing compound), and biosynthesis pathways with enrichment values >3 and p-values $<10^{-9}$. Similarities in pathway enrichment analysis were observed between F0 and F2, or F1 and F2 generations, but not F0 and F1.This is sensible when considering parents being under direct nicotine exposure, while their offspring originated from parents undergoing acute abstinence.

Of course, response to environmental stimuli systematically alters a myriad of factors. However, we funneled down our analysis and focused on five genes predicted to have a role in nicotine's mechanism of action across generations. The targets were regulated by the fourteen miRNAs whose expression levels were changed in more than one generation. We propose a model where *daf-4*, *daf-1*, *fos-1*, *cmk-1*, and *unc-30* are regulated by most of the fourteen miRNAs in a context dependent manner across generations. Based on annotations from WormBase (Yook et al., 2012), *daf-1* could mediate the reception of nicotine presence/absence

as it is expressed in the chemosensory neurons. *fos-1* and *cmk-1* mediate neuro-adaptation and remodeling in response to acute versus chronic exposure/withdrawal (Larson et al., 2011; Maze & Nestler, 2011; McPherson & Lawrence, 2007; Vonhoff, Kuehn, Blumenstock, Sanyal, & Duch, 2013). *daf-4* might explain the downstream phenotypic changes as it is involved in exit from dauer larval stage, body size determination, male tail patterning, egg laying, chemosensory neuron specification, thermo-tolerance and reproductive ability. It is important to consider miRNAs as working as a system where synergistic, additive, as well as antagonistic effects arise and are affected by nicotine dose, duration of exposure, generation, as well as previous ancestral experiences. The net effect is a generation-specific phenotype, characterized by shared as well as unique features.

References

Ajarem, J. S., & Ahmad, M. (1998). Prenatal nicotine exposure modifies behavior of mice through early development. *Pharmacol Biochem Behav., 59*(2), 313-318.

Ambros, V. (2003). MicroRNA pathways in flies and worms: growth, death, fat, stress, and timing. *Cell, 113*(6), 673-676. doi: S0092867403004288

Aukerman, M. J., & Sakai, H. (2003). Regulation of flowering time and floral organ identity by a MicroRNA and its APETALA2-like target genes. *Plant Cell, 15*(11), 2730-2741. doi: 10.1105/tpc.016238tpc.016238

Balaraman, S., Winzer-Serhan, U. H., & Miranda, R. C. (2012). Opposing actions of ethanol and nicotine on microRNAs are mediated by nicotinic acetylcholine receptors in fetal cerebral cortical-derived neural progenitor cells. *Alcohol Clin Exp Res, 36*(10), 1669-1677. doi: 10.1111/j.1530-0277.2012.01793.x

Beitel, G. J., Clark, S. G., & Horvitz, H. R. (1990). Caenorhabditis elegans ras gene let-60 acts as a switch in the pathway of vulval induction. *Nature, 348*(6301), 503-509. doi: 10.1038/348503a0

Benowitz, N. L. (1988). Pharmacologic Aspects of Cigarette Smoking and Nicotine Addiction. *NEJM., 319*(20), 1318-1330. doi: doi:10.1056/NEJM198811173192005

Brenner, J. L., Jasiewicz, K. L., Fahley, A. F., Kemp, B. J., & Abbott, A. L. (2010). Loss of individual microRNAs causes mutant phenotypes in sensitized genetic backgrounds in *C. elegans*. [Research Support, N.I.H., Extramural]. *Curr Biol, 20*(14), 1321-1325. doi: 10.1016/j.cub.2010.05.062

Brenner, S. (1974). The genetics of Caenorhabditis elegans. *Genetics, 77*(1), 71-94.

CDC. Morbidity and Mortality Weekly Report, (2008) (Date accessed: 12/08/2012) http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5745a3.htm

CDC. (2010). How Tobacco Smoke Causes Disease: The Biology and Behavioral Basis for Smoking-Attributable Disease: A Report of the Surgeon General. Atlanta (GA) Retrieved from http://www.ncbi.nlm.nih.gov/books/NBK53018/

Chen, C. Z., Li, L., Lodish, H. F., & Bartel, D. P. (2004). MicroRNAs modulate hematopoietic lineage differentiation. *Science, 303*(5654), 83-86. doi: 10.1126/science.10919031091903

Counotte, D. S., Spijker, S., Van de Burgwal, L. H., Hogenboom, F., Schoffelmeer, A. N., De Vries, T. J., . . . Pattij, T. (2009). Long-lasting cognitive deficits resulting from adolescent nicotine exposure in rats. *Neuropsychopharmacology, 34*(2), 299-306. doi: 10.1038/npp.2008.96

Crews, D., Gillette, R., Scarpino, S. V., Manikkam, M., Savenkova, M. I., & Skinner, M. K. (2012). Epigenetic transgenerational inheritance of altered stress responses. [Research Support, N.I.H., Extramural]. *PNAS., 109*(23), 9143-9148. doi: 10.1073/pnas.1118514109

Dani, J. A., & Heinemann, S. (1996). Molecular and cellular aspects of nicotine abuse. *Neuron, 16*(5), 905-908.

Eden, E., Navon, R., Steinfeld, I., Lipson, D., & Yakhini, Z. (2009). GOrilla: a tool for discovery and visualization of enriched GO terms in ranked gene lists. [Research Support, Non-U.S. Gov't]. *BMC Bioinformatics, 10*, 48. doi: 10.1186/1471-2105-10-48

El Marroun, H., Schmidt, M. N., Franken, I. H. A., Jaddoe, V. W. V., Hofman, A., van der Lugt, A., . . . White, T. (2014). Prenatal Tobacco Exposure and Brain Morphology: A

Prospective Study in Young Children. [Original Article]. *Neuropsychopharmacology, 39*(4), 792-800. doi: 10.1038/npp.2013.273

Feng, Z., Li, W., Ward, A., Piggott, B. J., Larkspur, E. R., Sternberg, P. W., & Xu, X. Z. (2006). A *C. elegans* model of nicotine-dependent behavior: regulation by TRP-family channels. *Cell, 127*(3), 621-633. doi: 10.1016/j.cell.2006.09.035

Furuhashi, H., Takasaki, T., Rechtsteiner, A., Li, T., Kimura, H., Checchi, P. M., . . . Kelly, W. G. (2010). Trans-generational epigenetic regulation of *C. elegans* primordial germ cells. *Epigenetics Chromatin, 3*(1), 15. doi: 10.1186/1756-8935-3-15

Heikkinen, L., Kolehmainen, M., & Wong, G. (2011). Prediction of microRNA targets in Caenorhabditis elegans using a self-organizing map. [Research Support, Non-U.S. Gov't]. *Bioinformatics, 27*(9), 1247-1254. doi: 10.1093/bioinformatics/btr144

Holloway, A. C., Cuu, D. Q., Morrison, K. M., Gerstein, H. C., & Tarnopolsky, M. A. (2007). Transgenerational effects of fetal and neonatal exposure to nicotine. [Research Support, Non-U.S. Gov't]. *Endocrine, 31*(3), 254-259.

Huang, d. W., Sherman, B., & Lempicki, R. (2009a). Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res, 37*(1), 1-13.

Huang, d. W., Sherman, B., & Lempicki, R. (2009b). Systematic and integrative analysis of large gene lists using DAVID Bioinformatics Resources. *Nature Protoc, 4*(1), 44-57.

Huang, W., & Li, M. D. (2009). Nicotine modulates expression of miR-140*, which targets the 3'-untranslated region of dynamin 1 gene (Dnm1). [Research Support, N.I.H., Extramural]. *Int J Neuropsychopharmacol, 12*(4), 537-546. doi: 10.1017/S1461145708009528

Kandel, D. B., & Chen, K. (2000). Extent of smoking and nicotine dependence in the United States: 1991-1993. *Nicotine Tob Res., 2*(3), 263-274.

Kassie, F., Jarcho, M., & Endalew, A. (2010). Abstract PR-06: Upregulation of microRNA-21 (miR-21) in human bronchial epithelial cells chronically exposed to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) plus nicotine and modulation of these effects by diindolylmethane. *Cancer Prev Res, 3*(1 Suppl), A51. doi: 10.1158/1940-6207.PREV-09-PR-06

Kato, M., de Lencastre, A., Pincus, Z., & Slack, F. J. (2009). Dynamic expression of small non-coding RNAs, including novel microRNAs and piRNAs/21U-RNAs, during Caenorhabditis elegans development. *Genome Biol, 10*(5), R54. doi: 10.1186/gb-2009-10-5-r54

Kim, V. N. (2005). MicroRNA biogenesis: coordinated cropping and dicing. *Nat Rev Mol Cell Biol, 6*(5), 376-385. doi: 10.1038/nrm1644

Kolokotroni, K. Z., Rodgers, R. J., & Harrison, A. A. (2012). Effects of chronic nicotine, nicotine withdrawal and subsequent nicotine challenges on behavioural inhibition in rats. [Research Support, Non-U.S. Gov't]. *Psychopharmacology (Berl), 219*(2), 453-468. doi: 10.1007/s00213-011-2558-z

Larson, E. B., Graham, D. L., Arzaga, R. R., Buzin, N., Webb, J., Green, T. A., . . . Self, D. W. (2011). Overexpression of CREB in the nucleus accumbens shell increases cocaine reinforcement in self-administering rats. [Research Support, N.I.H., Extramural]. *J Neurosci., 31*(45), 16447-16457. doi: 10.1523/JNEUROSCI.3070-11.2011

Lau, N. C., Lim, L. P., Weinstein, E. G., & Bartel, D. P. (2001). An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. *Science, 294*(5543), 858-862. doi: 10.1126/science.1065062

Lee, R. C., Feinbaum, R. L., & Ambros, V. (1993). The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell, 75*(5), 843-854. doi: 0092-8674(93)90529-Y

Lim, L. P., Lau, N. C., Weinstein, E. G., Abdelhakim, A., Yekta, S., Rhoades, M. W., . . . Bartel, D. P. (2003). The microRNAs of Caenorhabditis elegans. *Genes Dev, 17*(8), 991-1008. doi: 10.1101/gad.1074403

Maritz, G. S. (2013). Perinatal exposure to nicotine and implications for subsequent obstructive lung disease. [Review]. *Paediatr Respir Rev., 14*(1), 3-8. doi: 10.1016/j.prrv.2012.03.006

Matta, S. G., Balfour, D. J., Benowitz, N. L., Boyd, R. T., Buccafusco, J. J., Caggiula, A. R., . . . Zirger, J. M. (2007). Guidelines on nicotine dose selection for in vivo research. *Psychopharmacology (Berl), 190*(3), 269-319. doi: 10.1007/s00213-006-0441-0

Maze, I., & Nestler, E. J. (2011). The epigenetic landscape of addiction. [Review]. *Ann N Y Acad Sci., 1216*, 99-113. doi: 10.1111/j.1749-6632.2010.05893.x

McManus, M. T. (2003). MicroRNAs and cancer. *Semin Cancer Biol, 13*(4), 253-258.

McPherson, C. S., & Lawrence, A. J. (2007). The nuclear transcription factor CREB: involvement in addiction, deletion models and looking forward. *Curr Neuropharmacol., 5*(3), 202-212. doi: 10.2174/157015907781695937

Ng, T. K., Carballosa, C. M., Pelaez, D., Wong, H. K., Choy, K. W., Pang, C. P., & Cheung, H. S. (2013). Nicotine Alters MicroRNA Expression and Hinders Human Adult Stem Cell Regenerative Potential. *Stem Cells Dev., 22*(5), 781-790. doi: 10.1089/scd.2012.0434

Riddle, D. L., Blumenthal, T., & B.J., M. [*C. elegans* II. 2nd edition.] (CSHL Press., 1997) http://www.ncbi.nlm.nih.gov/books/NBK20086/.

Saeed, A., NK, B., JC, B., W, L., V, S., EA, H., & al., e. (2006). TM4 microarray software suite. *Methods Enzymol., 411*, 134-193.

Samaha, A. N., Yau, W. Y., Yang, P., & Robinson, T. E. (2005). Rapid delivery of nicotine promotes behavioral sensitization and alters its neurobiological impact. *Biol Psychiatry., 57*(4), 351-360. doi: 10.1016/j.biopsych.2004.11.040

Shan, H., Zhang, Y., Lu, Y., Pan, Z., Cai, B., Wang, N., . . . Yang, B. (2009). Downregulation of miR-133 and miR-590 contributes to nicotine-induced atrial remodelling in canines. [Research Support, Non-U.S. Gov't]. *Cardiovasc Res., 83*(3), 465-472. doi: 10.1093/cvr/cvp130

Shin, V. Y., Jin, H., Ng, E. K., Cheng, A. S., Chong, W. W., Wong, C. Y., . . . Chu, K. M. (2011). NF-kappaB targets miR-16 and miR-21 in gastric cancer: involvement of prostaglandin E receptors. [Research Support, Non-U.S. Gov't]. *Carcinogenesis, 32*(2), 240-245. doi: 10.1093/carcin/bgq240

Skinner, M. K. (2008). What is an epigenetic transgenerational phenotype? F3 or F2. [Review]. *Reprod Toxicol, 25*(1), 2-6. doi: 10.1016/j.reprotox.2007.09.001

Skjei, K. L., & Markou, A. (2003). Effects of repeated withdrawal episodes, nicotine dose, and duration of nicotine exposure on the severity and duration of nicotine withdrawal in rats. *Psychopharmacology (Berl), 168*(3), 280-292. doi: 10.1007/s00213-003-1414-1

Slotkin, T. A. (2002). Nicotine and the adolescent brain: insights from an animal model. *Neurotoxicol Teratol., 24*(3), 369-384.

Smoot, M. E., Ono, K., Ruscheinski, J., Wang, P. L., & Ideker, T. (2011). Cytoscape 2.8: new features for data integration and network visualization. [Research Support, N.I.H., Extramural]. *Bioinformatics, 27*(3), 431-432. doi: 10.1093/bioinformatics/btq675

Sobkowiak, R., Kowalski, M., & Lesicki, A. (2011). Concentration- and time-dependent behavioral changes in Caenorhabditis elegans after exposure to nicotine. *Pharmacol Biochem Behav., 99*(3), 365-370. doi: 10.1016/j.pbb.2011.05.019

Sulston, J., & Hodgkin, J. (1988). The Nematode Caenorhabditis elegans, W.B. Wood, ed. *New York: Cold Spring Harbor Laboratory Press*, p. 587.

Taki, F., Pan, X., & Zhang, B. (2013). Nicotine Exposure Caused Significant Transgenerational Heritable Behavioral Changes in Caenorhabditis elegans. *Excli J., 12*(1611-2156), 793-806. doi: http://www.excli.de/vol12/Zhang_10092013_proof.pdf

Taki, F. A., Pan, X., & Zhang, B. (2013). Chronic nicotine exposure systemically alters microRNA expression profiles during post-embryonic stages in Caenorhabditis elegans. *J Cell Physiol, 229*(1), 79-89. doi: 10.1002/jcp.24419

Thomas, L. T., Welsh, L., Galvez, F., & Svoboda, K. R. (2009). Acute nicotine exposure and modulation of a spinal motor circuit in embryonic zebrafish. *Toxicol Appl Pharmacol., 239*(1), 1-12. doi: 10.1016/j.taap.2008.08.023

USDHHS. [The Health Consequences of Smoking: Nicotine Addiction] [1-618] (Center for Health Promotion and Education. Office on Smoking and Health, 1988).

Vonhoff, F., Kuehn, C., Blumenstock, S., Sanyal, S., & Duch, C. (2013). Temporal coherency between receptor expression, neural activity and AP-1-dependent transcription regulates Drosophila motoneuron dendrite development. [Research Support, N.I.H., Extramural]. *Development, 140*(3), 606-616. doi: 10.1242/dev.089235

Wada, E., Wada, K., Boulter, J., Deneris, E., Heinemann, S., Patrick, J., & Swanson, L. W. (1989). Distribution of alpha 2, alpha 3, alpha 4, and beta 2 neuronal nicotinic receptor subunit mRNAs in the central nervous system: a hybridization histochemical study in the rat. *J Comp Neurol., 284*(2), 314-335. doi: 10.1002/cne.902840212

WHO. Tobacco, (2012) (Date accessed: 24/03/2012) http://www/who.int/mediacentre/factsheets/fs339/en/.

WHO. World Health Statistics.Global Health Observatory, (2012) (Date accessed: 24/03/2012) http://www.who.int/gho/publications/world_health_statistics/EN_WHS2012_Full.pdf.

Yook, K., Harris, T. W., Bieri, T., Cabunoc, A., Chan, J., Chen, W. J., . . . Sternberg, P. W. (2012). WormBase 2012: more genomes, more data, new website. *Nucleic Acids Res., 40*(D1), D735-D741. doi: 10.1093/nar/gkr954

Zhang, B., Pan, X., Cobb, G. P., & Anderson, T. A. (2007). microRNAs as oncogenes and tumor suppressors. *Developmental Biology, 302*(1), 1-12. doi: http://dx.doi.org/10.1016/j.ydbio.2006.08.028

Zhang, B., Wang, Q., & Pan, X. (2007). MicroRNAs and their regulatory roles in animals and plants. [Review]. *J Cell Physiol., 210*(2), 279-289. doi: 10.1002/jcp.20869

Table 2.1: The effect of parental post-embryonic nicotine exposure on miRNA expression levels in L4 worms belonging to the F1 generation. (* and β denote p-values<0.05 in comparison to control).

	F1 Low				F1 High			
	P value	MFC	±	SE	P value	MFC	±	SE
***miR1**	0.813	0.12	±	0.44	0.020	-0.60	±	0.09
***miR1019**	0.321	0.92	±	0.78	0.005	1.58	±	0.29
***miR1828**	0.517	0.68	±	0.93	0.025	-0.47	±	0.11
***miR1829a**	0.637	-0.29	±	0.53	0.006	-0.75	±	0.06
***miR1829b**	0.009	-0.25	±	0.04	0.001	-0.68	±	0.06
***miR1829c**	0.025	-0.37	±	0.11	0.027	-0.53	±	0.09
***miR2208aSt**	0.883	0.12	±	0.73	0.041	-0.66	±	0.14
***miR2212St**	0.397	1.48	±	1.50	0.037	-0.51	±	0.17
***miR2218a**	0.581	0.37	±	0.57	0.044	-0.50	±	0.17
***miR236**	0.313	0.38	±	0.28	0.010	0.81	±	0.14
***miR242**	0.758	0.19	±	0.53	0.024	-0.47	±	0.07
β*miR253	0.000	-0.97	±	0.01	0.000	-0.97	±	0.00
β*miR254	0.006	3.47	±	0.49	0.018	6.41	±	0.87
βmiR255	0.001	-0.71	±	0.09	0.881	-0.10	±	0.60
***miR257**	0.117	3.70	±	1.69	0.034	4.81	±	1.30
***miR260**	0.075	3.03	±	1.13	0.023	5.58	±	0.86
***miR262**	0.064	0.59	±	0.20	0.014	0.58	±	0.07
***miR353**	0.437	0.33	±	0.39	0.016	0.67	±	0.17
***miR36**	0.175	1.28	±	0.72	0.014	2.90	±	0.56
βmiR41	0.000	-0.53	±	0.01	0.586	0.18	±	0.30
***miR42**	0.293	-0.30	±	0.21	0.013	-0.48	±	0.11
***miR53**	0.429	-0.24	±	0.25	0.001	-0.52	±	0.06
***miR56St**	0.531	-0.25	±	0.33	0.005	-0.62	±	0.05
βmiR66	0.040	0.70	±	0.20	0.091	1.01	±	0.41
***miR71**	0.396	-0.29	±	0.28	0.011	-0.62	±	0.06
β*miR785	0.036	-0.54	±	0.10	0.002	-0.52	±	0.03
βmiR786	0.002	-0.65	±	0.06	0.437	-0.21	±	0.25
***miR786St**	0.700	0.18	±	0.40	0.022	-0.45	±	0.07
βmiR793	0.031	1.21	±	0.22	0.685	-0.07	±	0.15
βmiR794	0.029	-0.54	±	0.14	0.242	-0.26	±	0.16
***miR85**	0.858	0.06	±	0.29	0.010	-0.59	±	0.06

Table 2.2: The effect of parental post-embryonic nicotine exposure on miRNA expression levels in L4 worms belonging to the F2 generation. (* and β denote p-values<0.05 in comparison to control).

	F2 Low				F2 High			
	P value	MFC	±	SE	P value	MFC	±	SE
***miR1**	0.636	0.24	±	0.44	0.005	2.00	±	0.35
***miR1828**	0.841	0.06	±	0.26	0.046	-0.46	±	0.10
***miR2212St**	0.696	0.10	±	0.23	0.001	0.50	±	0.02
***miR2218a**	0.843	0.07	±	0.33	0.013	0.96	±	0.11
***miR2220**	0.137	-0.40	±	0.17	0.001	0.75	±	0.09
β*miR232	0.000	-0.61	±	0.05	0.015	-0.59	±	0.07
***miR235**	0.003	0.64	±	0.10	0.921	0.03	±	0.27
β*miR239a	0.006	-0.57	±	0.11	0.000	-0.69	±	0.05
β*miR240	0.012	-0.61	±	0.07	0.001	-0.59	±	0.02
β*miR244	0.000	-1.00	±	0.00	0.000	-1.00	±	0.00
βmiR255	0.009	-0.50	±	0.05	0.970	0.00	±	0.11
βmiR51	0.043	0.55	±	0.19	0.435	0.29	±	0.29
β*miR53	0.000	-1.00	±	0.00	0.000	-1.00	±	0.00
βmiR790	0.041	0.48	±	0.16	0.054	0.35	±	0.08
β*miR80	0.027	0.81	±	0.14	0.035	0.57	±	0.11
***miR800**	0.110	0.38	±	0.14	0.049	0.52	±	0.19

Table 2.3: A summary of genes clustered with high enrichment using Gene functional annotation via DAVID. Worm annotations were obtained from WormBase (Yook et al., 2012).

Gene Group 1	**Enrichment Score: 21.4**
Official gene symbol	**Function**
unc-30 (UNCoordinated)	• a homeodomain-containing protein • orthologous to the Pitx family of homeodomain transcription factors • controls the terminal differentiation of all 19 type D GABA-ergic motor neurons during development
skn-1 (SKiNhead)	• a bZip transcription factor orthologous to the mammalian Nrf (Nuclear factor-erythroid-related factor) transcription factors • required for specification of the EMS blastomere, a mesendodermal precursor that gives rise to pharyngeal, muscle, and intestinal cells during early embryogenesis • functions in the p38 MAPK pathway to regulate the oxidative stress response and in parallel to DAF-16/FOXO in the DAF-2-mediated insulin/IGF-1-like signaling pathway to regulate adult lifespan
nhr-49 (Nuclear Hormone Receptor family)	• a nuclear hormone receptor (NHR) related to the mammalian HNF4 (hepatocyte nuclear factor 4) family of NHRs • a key regulator of fat metabolism and lifespan by regulating induction of beta-oxidation genes upon food deprivation and activation of stearoyl-CoA desaturase in fed animals, respectively • activates transcription in conjunction with the MDT-15 mediator subunit with which it physically interacts.
fos-1 (FOS B-Zip transcription factor homolog)	• encodes two basic region-leucine zipper (bZip) transcription factors, FOS-1A and FOS-1B, that are the sole *C. elegans* ortholog of the fos bZip transcription factor family • required cell autonomously in the gonadal anchor cell for basement-membrane removal and subsequent anchor cell invasion of the vulval epithelium
nfi-1 (Nuclear Factor I family)	• encodes the *C. elegans* ortholog of the Nuclear Factor I (NFI) family of transcription factors in *C. elegans*, • required for locomotion, egg laying, pharyngeal pumping, and wild-type adult lifespan
vab-3 (Variable ABnormal morphology)	• a homeodomain protein (Pax-6 ortholog) • required for proper patterning of anterior (head) hypodermal cells • required for epidermal morphogenesis, epidermal cell fates, gonad cell migration and the development of sensory structures in the male tail
Gene Group 2	**Enrichment Score: 11.3**
Official gene symbol	**Function**
daf-4 (abnormal DAuer Formation)	• a transmembrane serine/threonine kinase (sole ortholog of the type II transforming growth factor-beta (TGF-b) receptors) • required for several biological processes, including entry into and exit from the dauer larval stage, body size determination, male tail patterning, egg laying, chemosensory neuron specification, and increased thermotolerance

	• regulates reproductive aging, via the TGF-beta Sma/Mab pathway
daf-1 (abnormal DAuer Formation)	• a TGF-beta type I receptor homolog • required for the regulation of dauer formation by environmental signals through the ASI chemosensory neuron
cmk-1 (CaM Kinase)	• a Ca+2/calmodulin-dependent protein kinase I (CaMK1) • required, cell autonomously and downstream of the cyclic nucleotide-gated channel TAX-4, for several aspects of AFD thermosensory neuron differentiation, including expression of the gcy-8 guanylyl cyclase and nhr-38 nuclear hormone receptor genes and morphology of the AFD sensory endings • required for normal thermosensory behavior • positively regulate the transcriptional activity of endogenous CREB
pek-1 (human PERK kinase homolog)	• a predicted transmembrane protein kinase (orthologous to human eukaryotic translation initiation factor 2-alpha kinase 3 (EIF2AK3)) • strongly expressed in intestinal cells • required for the unfolded protein response (UPR) that counteracts cellular stress induced by accumulation of unfolded proteins in the endoplasmic reticulum (ER) • may phosphorylate eIF2alpha and inhibit protein synthesis in response to endogenous ER stress
sma-6 (SMAll)	• a serine/threonine protein kinase (ortholog of type I TGF-beta receptors) • required for regulating body length and for proper development of the male tail • regulates reproductive aging • sufficient for body length regulation

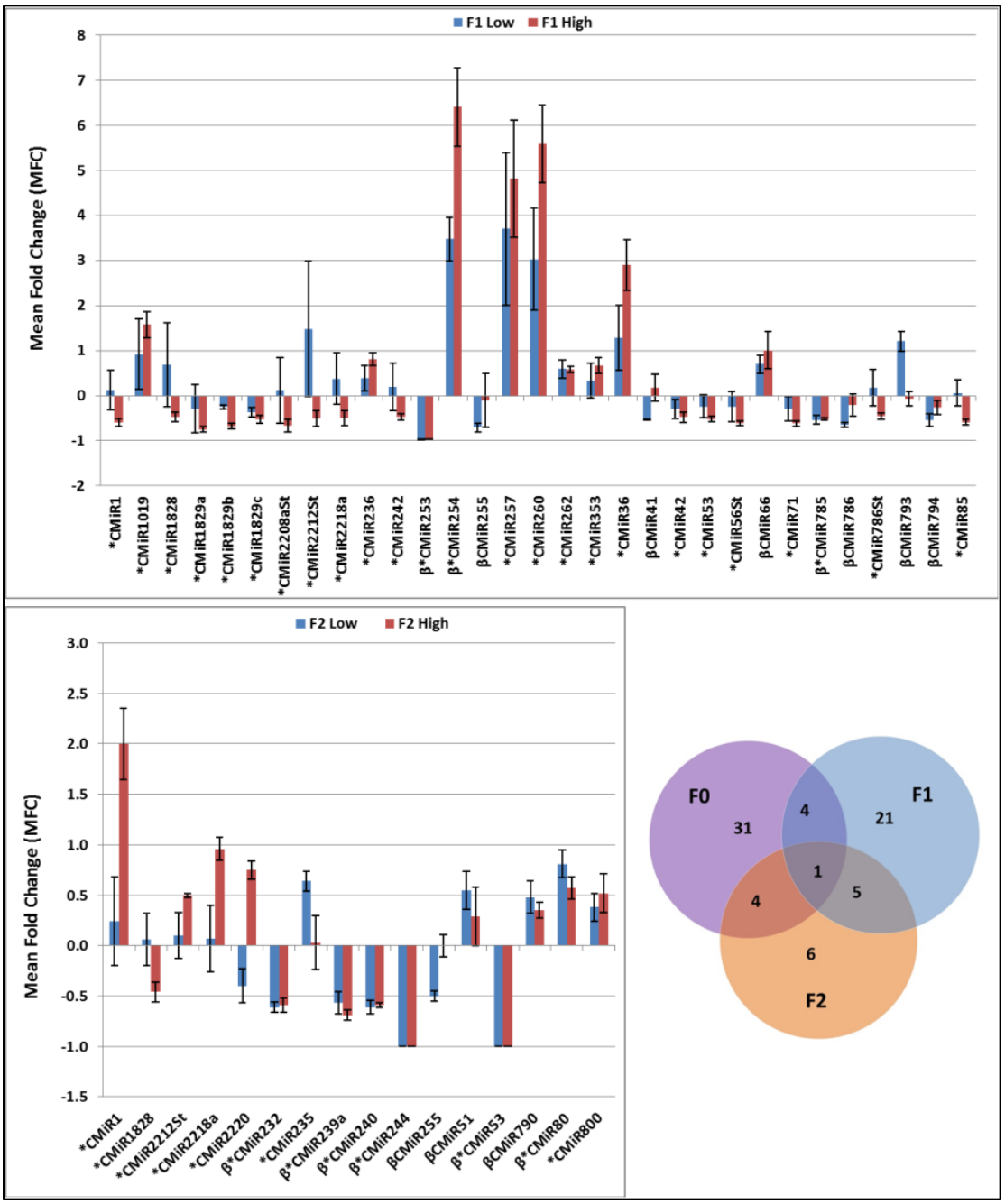
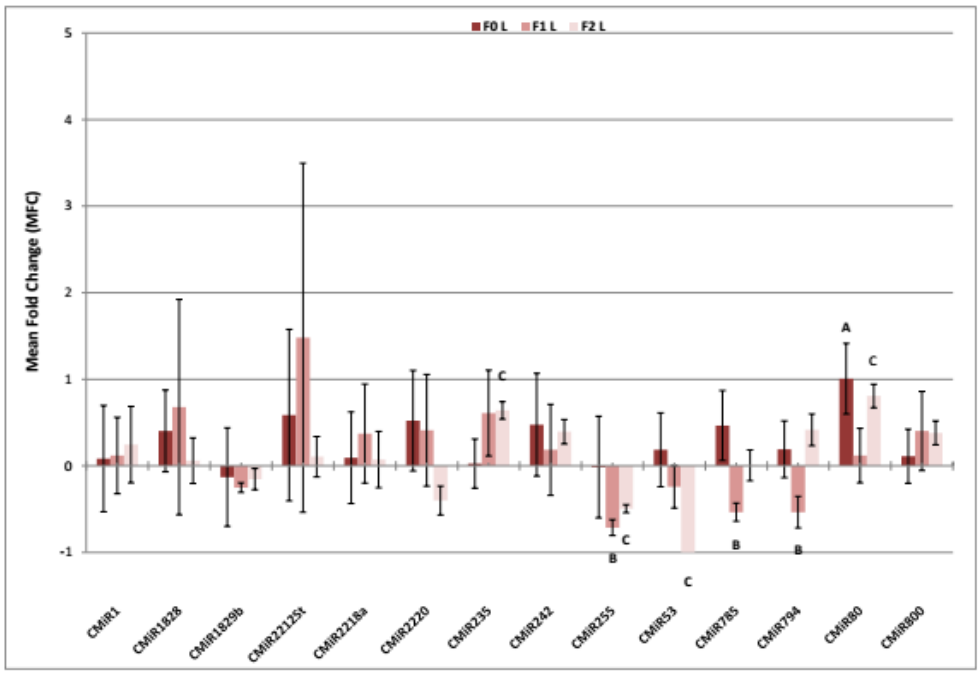


Figure 2.1: Nicotine altered the miRNA expression profiles across generations in a dose-dependent manner. (a) Nicotine significantly altered the expression levels of 31 miRNAs in the F1 worm population. (b) Nicotine significantly altered the expression levels of 16 miRNAs in the F2 worm population. (c) A Venn diagram showing the number of the miRNAs with differentially altered expression levels shared in L4 larvae belonging to the three generations (F0, F1, and F2). $P<0.05$. [*, β denote statistically significant changes in response to high (20mM) and low (20µM) nicotine concentrations, respectively). All comparisons were based on control.

(A)



(B)

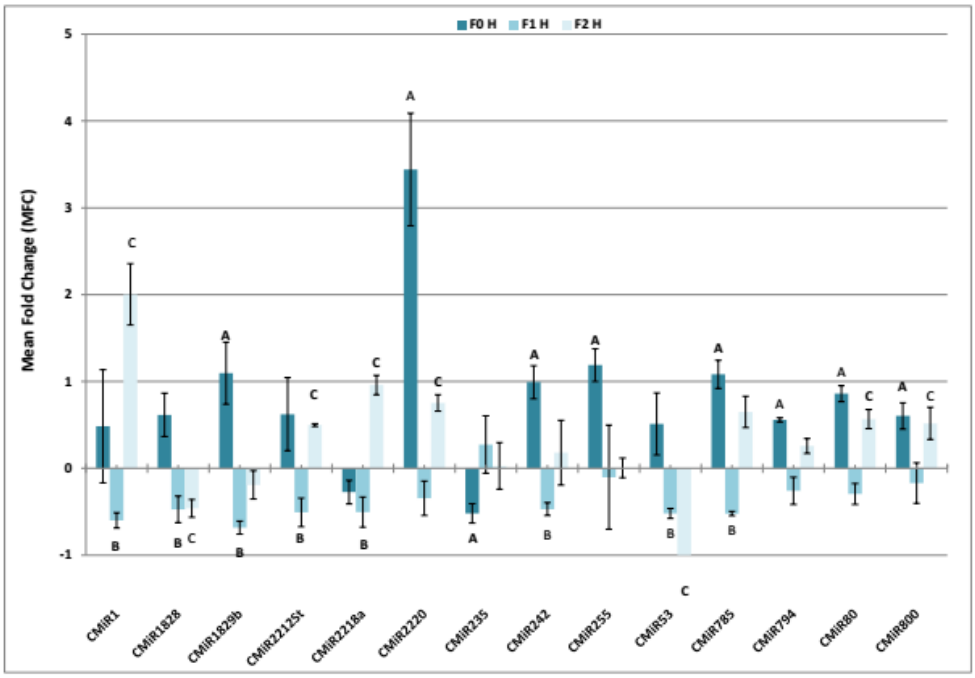


Figure 2.2: Nicotine altered the expression levels of 14 miRNAs common to at least two generations. Different colors represent different treatment groups belonging to each of the three generations. From Left to right, bars represent Mean Fold Changes (MFC) for miRNAs belonging to (A) F0 L, F1 L, F2, L, and (B) F0 H, F1 H, and F2 H. L stands for low, and H stands for high nicotine dose. Data labels A, B, and C denote p-values <0.05 in comparison to control in F0, F1, and F2, respectively.

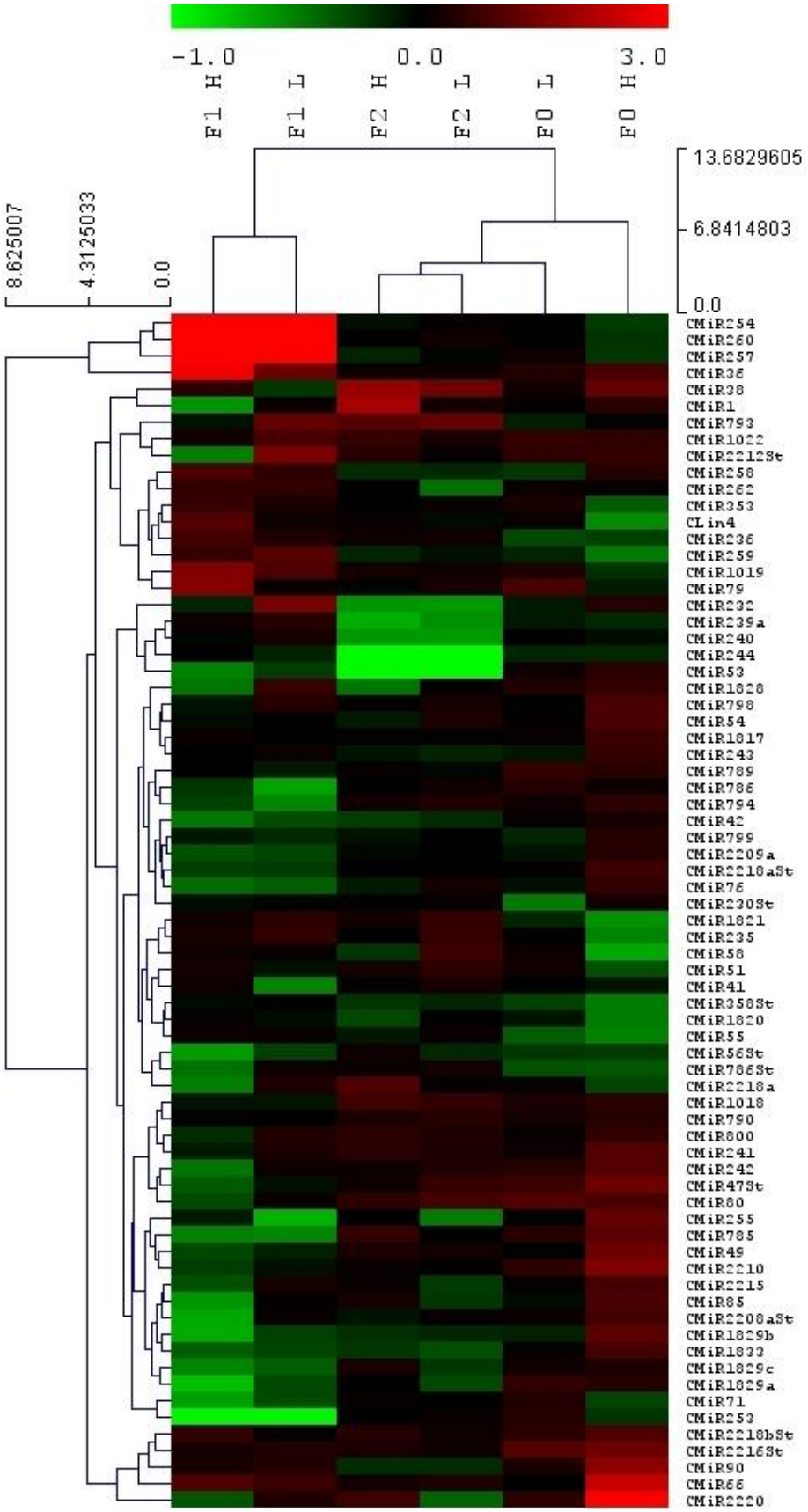


Figure 2.3: Nicotine exposure limited to L4 of F0 generation caused differential clustering in three L4 generations in *C. elegans* (N2). Unsupervised hierarchical clustering based on Euclidean distance and complete linkage with optimization was performed for samples (3 biological replicates per treatment group) and miRNAs. Each cell represents a MFC compared to control (Mean Fold Change: $2(\Delta\Delta CT)$ -1). In the figures, color red, green, and black represent up-regulation, down-regulation and no change with respect to control, respectively. Graph was done using Mev software (Saeed et al., 2006).

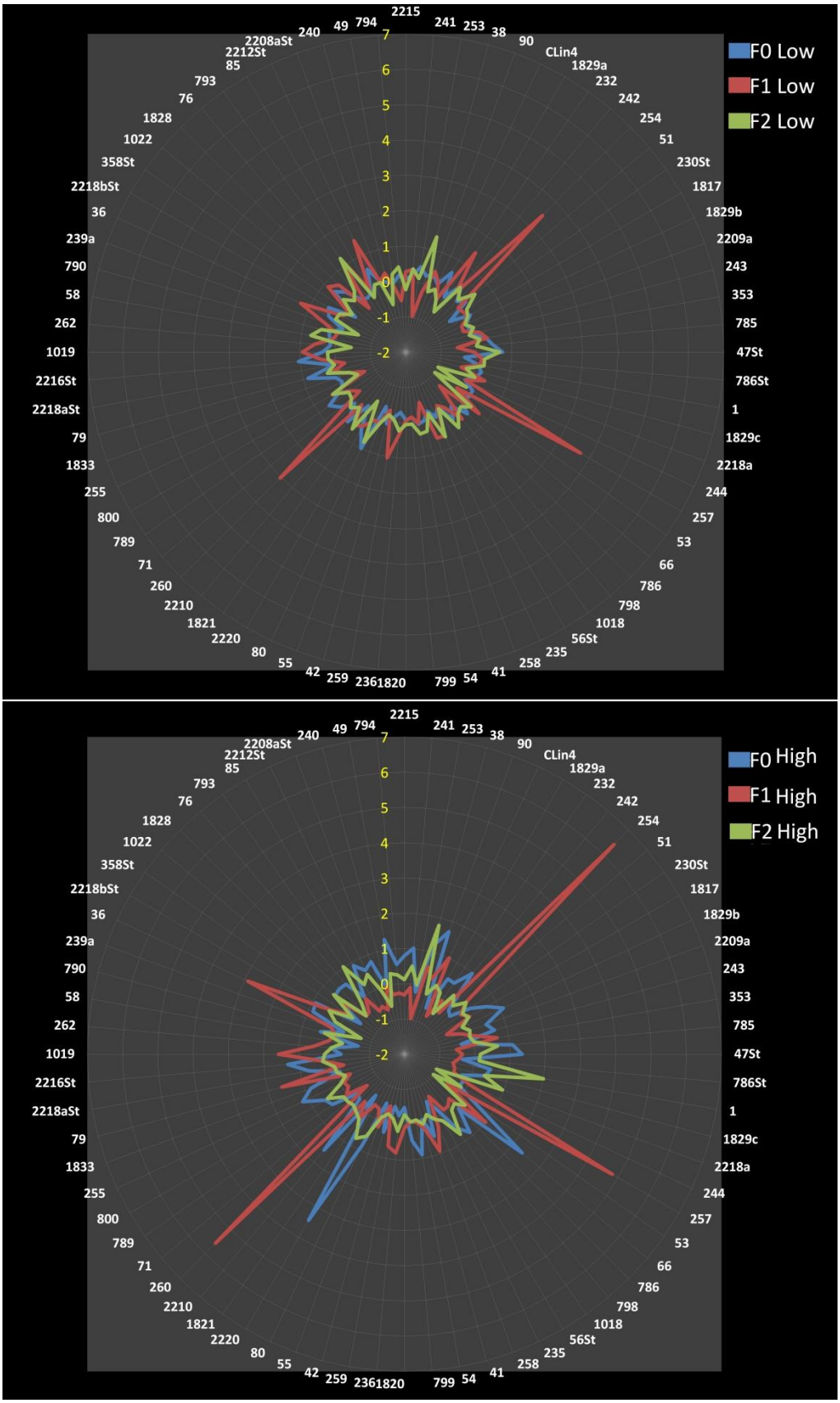


Figure 2.4: Radar graph showing general patterns of miRNA MFCs across generations in response to nicotine treatment limited to the post-embryonic stage of the parent F0 generations in *C. elegans* (N2). The MFCs of 72 miRNAs were used for the low treatment groups (20µM) (a) and high treatment groups (20mM) (b). Each concentric rim represents 1 fold increase or decrease (2(ΔΔCT) -1) from 0. Different generations are presented by different colors. The input miRNA list had the same order among each treatment group (low and high nicotine concentration) to allow for comparisons.

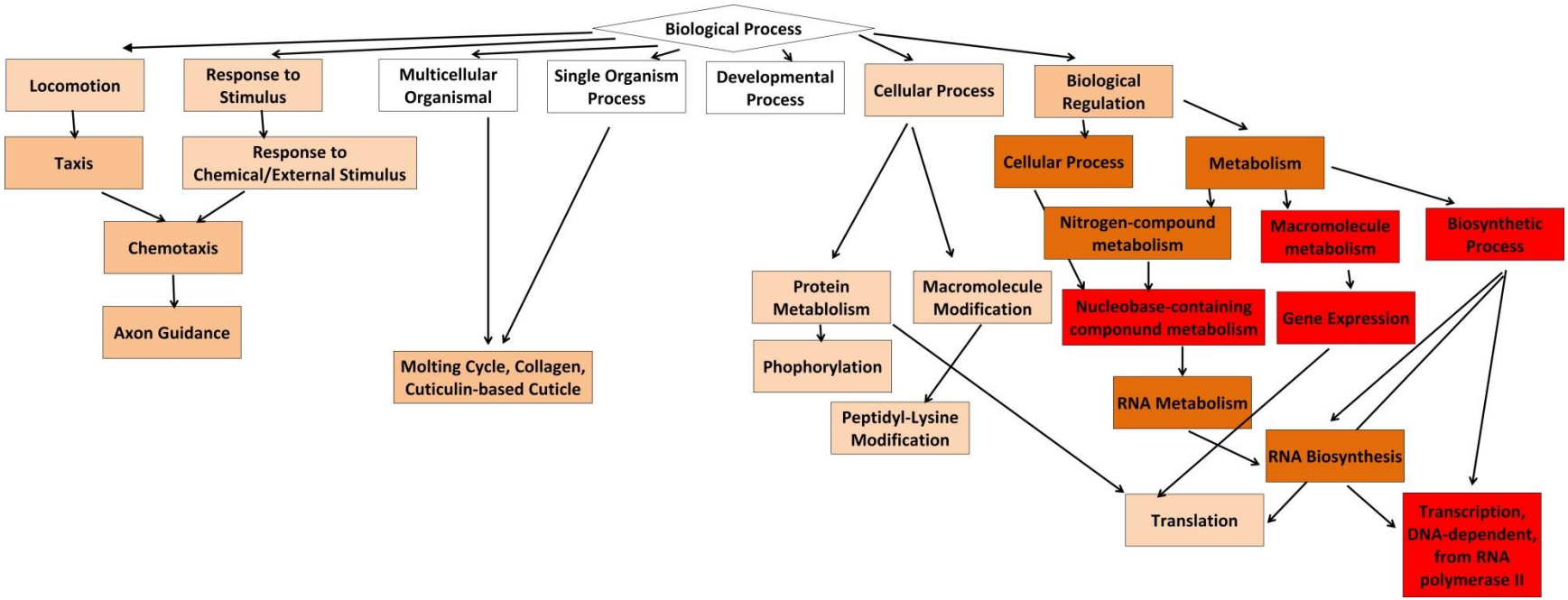
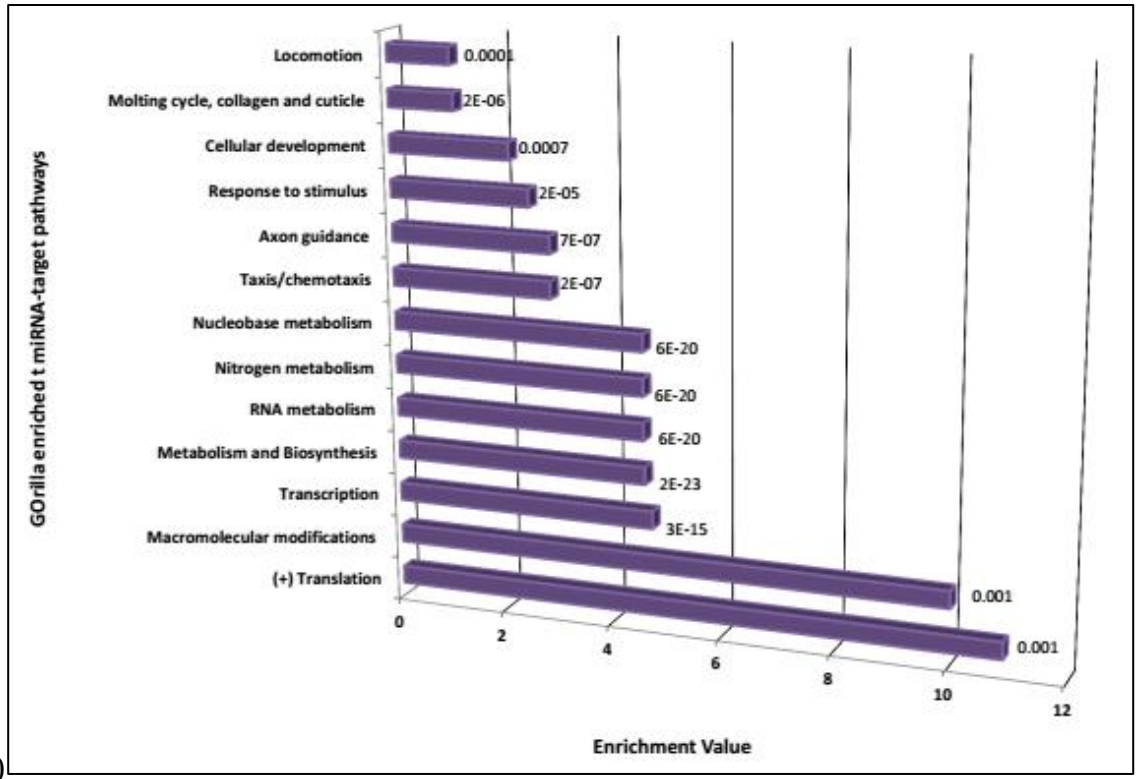


Figure 2.5: Directed acyclic graph (DAG) performed by GOrilla. 14 miRNAs that were differentially expressed in at least 2 generations were used for target prediction. Only genes belonging to enriched clusters in DAVID were used to prepare the single gene ranked list for GOrilla input as described in the text. The Graph shows the relationships among the enriched pathways targeted by miRNAs altered in response to nicotine treatment. Colors represent P-values. From white to orange/red, p-values range from $>10^{-3}$ to $<10^{-9}$.

(A)



(B)

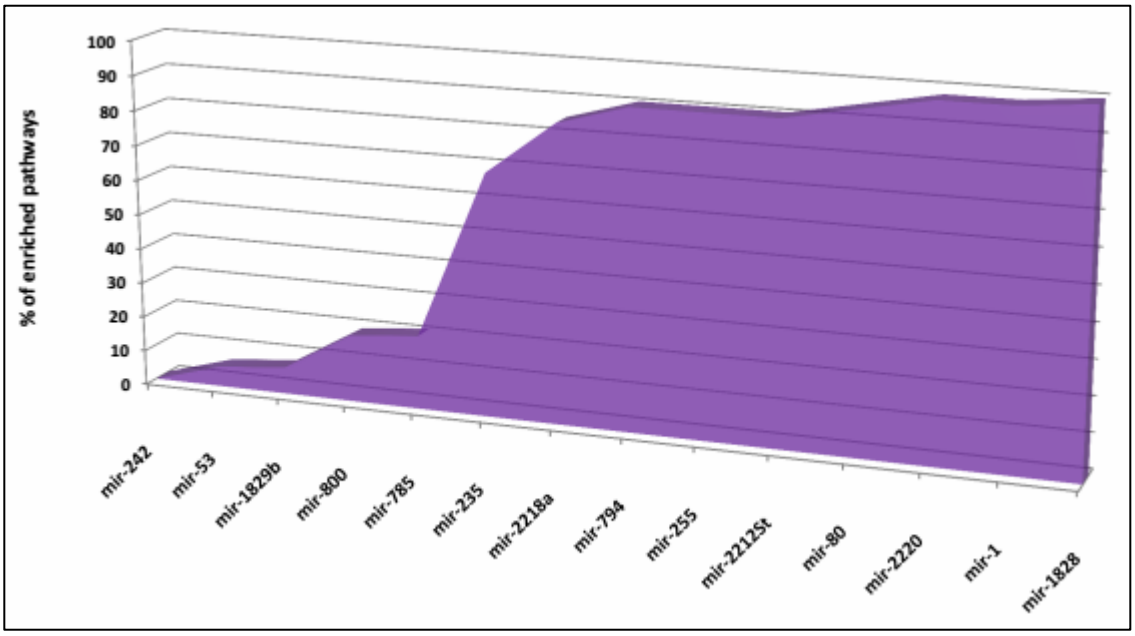
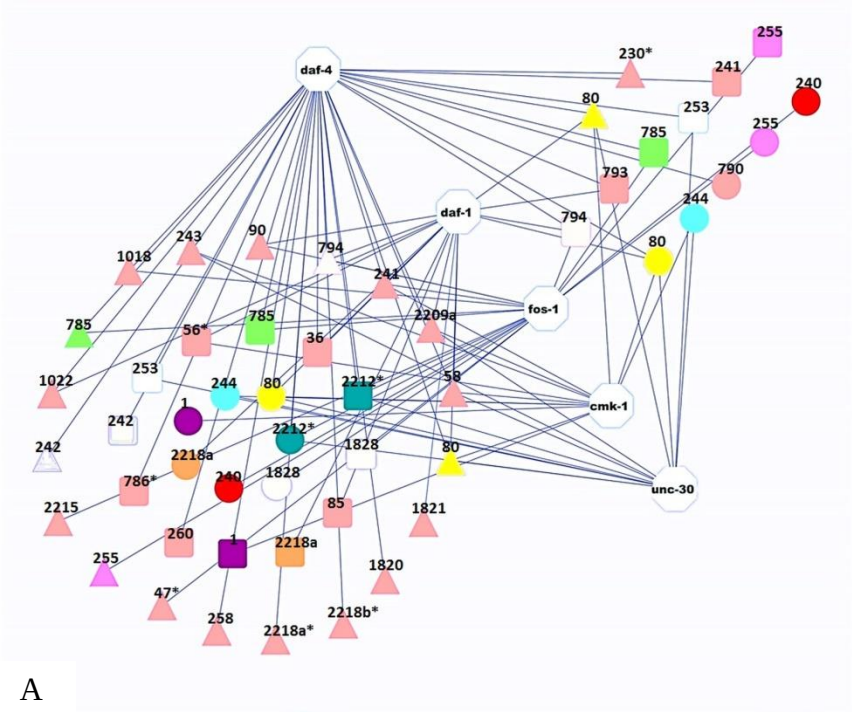
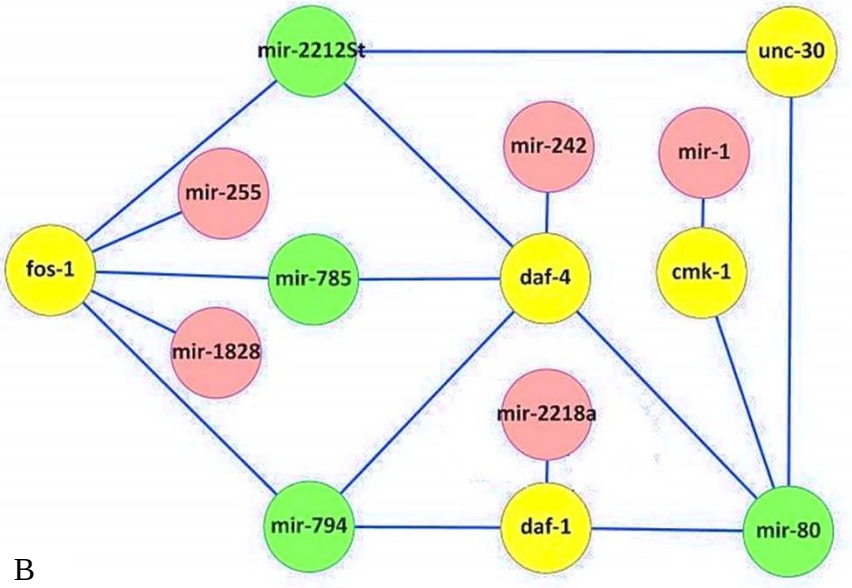


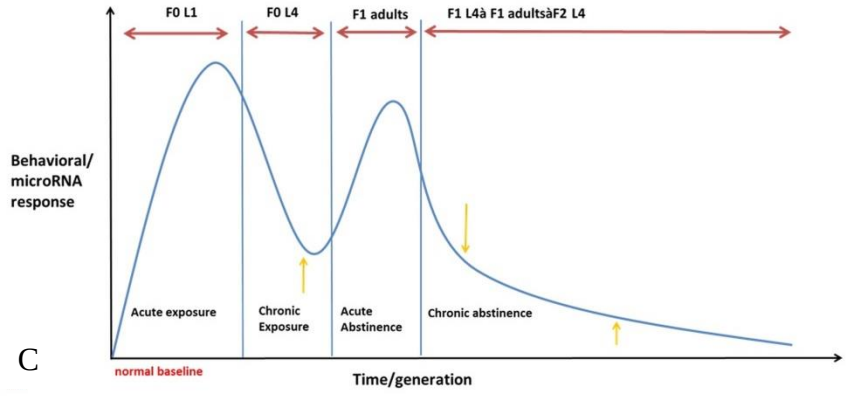
Figure 2.6: Analyzing the roles of the 14 commonly altered miRNAs in the enriched pathways. (a) A summary of the pathways predicted to be altered in response to nicotine treatment across the three generations. The data labels represent the respective p-values for each enriched process. (b) Variation in miRNA regulation. Some pathways are commonly regulated by 2 or more miRNAs while others are specific to 1 miRNA. On the other hand, there was an apparent difference in the % of pathways predicted to be regulated by individual miRNAs.



A



B



C

Figure 2.7: Nicotine exerts its transgenerational effect via five enriched gene candidates. (A) A network showing relationships between differentially altered miRNAs with gene candidates in each generation. Triangles denote worms of the F0 generations, while squares and circles denote worms of the F1 and F2 generations, respectively. The five genes candidates are in hexagons in the middle. The low concentration treatment groups are shown on the up-right side, while the high concentration treatment groups are shown in the left bottom side. (B) A simplified network showing interactions between only commonly altered miRNAs and the candidate genes. Candidate genes are in yellow. miRNAs targeting more than one gene are in green. (C) A hypothesized model to explain the nicotine-induced miRNA expression profiles and behaviors as a function of duration of exposure or abstinence and previous parental life experiences.

Chapter III

MS80-mediated resistance against nicotine-induced pharyngeal pumping inhibition

Abstract

Nicotine is the major addictive chemical in tobacco smoke of which exposure remains differentially yet highly prevalent during the neonatal and adolescent periods. This early exposure is associated with direct effects like delayed development, sudden infant death, and arrhythmias; however, the molecular mechanisms behind those health disparities remain unknown. Based on our prior transcriptomic data and bioinformatics analysis, we aimed to focus on the interplay of mpk-1, miR-80, and sir-2.1 (MS80) in nicotine-induced early onset diseases using the model organism *C. elegans*. Worms of different genetics backgrounds were treated with nicotine from L1 to early L4 larval stages prior to sexual maturity and then were used for growth and behavioral assays. Our length and seam cell count showed that nicotine arrested the worms at the L1 stage and this effect was not rescued with MS80. Nicotine also inhibited pharyngeal pumping in the worms yet this effect could be dissociated from the arrested development. Nicotine-induced pumping inhibition was mediated by MS80 as worms with loss-of-function mutations in mpk-1, miR-80, or sir-2.1 were resistant to nicotine-induced reduction in pumping rate. On the other hand, pharyngeal mpk-1 overexpression mirrored the phenotype in N2 worms, while it sensitized miR-80 (*lof*) and sir-2.1 (*lof*) worms to nicotine-induced pumping inhibition. Interestingly, co-treatment with U0126 influenced pumping rate in miR-80 and sir-2.1 backgrounds and this could be mediated by the increased activation of aak-2/AMPK downstream of nicotine-induced reduction in serotonin signaling. So, while the mechanism behind nicotine-induced delay in development remains unknown, our study is the first to report a relationship between mpk-1, sir-2.1, and miR-80 in mediating nicotine-induced hyperactivation of

neuromotor signaling and the consequential muscular tetanus during the early developmental periods.

Keywords: Nicotine, MAPK, ERK1/2, mpk-1, sirtuins, SIRT1, sir-2.1, miR-80, C. elegans larvae, neonates, adolescents, early on-set, neuromotor signaling, developmental delay

Introduction

Nicotine, the major addictive component of tobacco, is associated with a wide spectrum of cardiac and respiratory arrhythmias (D'Alessandro, Boeckelmann, Hammwhoner, & Goette, 2012). In addition, smoking altered respiratory sinus arrhythmia and heart rate in adolescents (Conrad, Gorka, & Kassel, 2015). However, the pathophysiological mechanisms for nicotine-induced arrhythmias are poorly understood. Here, we took advantage of the genetic tools and behavioral assays available for the model organism *C. elegans*. The *C. elegans* pharynx is an alimentary tissue that is considered a prototype to the human heart (Olson, 2006). Both have similar electrical activity that is the output of several action potentials induced by a myriad of physiological channels. Though evolved for a different function which is feeding, its proper function is required for proper growth and development. Normal pharyngeal function involves the right timing, pumping frequency, and downstream peristalsis (Avery & You, 2012). In wild type worms, the pharynx pumps rhythmically and rarely pauses and this behavior is impacted by certain environmental exposure (e.g. chemicals, food).Thus, we employed this phenotype as a proxy to study the molecular mechanism for nicotine induced arrhythmias.

Previously, we profiled global microRNA expressions across three generations in response to post-embryonic nicotine exposure (F. A. Taki et al., 2014). We identified 14 miRNAs that were impacted in at least two generations. Among them, we were interested in miR-80 which was upregulated in the parent (F0) generation as well as in the grand-offspring (F2) generation. Based on our previous bioinformatics analysis, fos-1 and cmk-1 were among the most common transcripts predicted to be targeted by the 14 transgenerational miRNAs (F. A. Taki et al., 2014). cmk-1 regulates CREB (cAMP responsive element). CREB interacts with its coactivator CREB-binding protein (CBP) which is a histone acetyl transferase that activates

transcription and has been shown to be a target of miR-80 in global regulation of metabolism (Vora et al., 2013). More so, fos-1 is orthologous to the fos bZip transcription factor family. Both cFos and CREB have been reported to mediate responses to drugs of abuse like cocaine through sirtuin and miRNA dependent mechanisms (Ferguson et al., 2013; Gao et al., 2010; Robison & Nestler, 2011). In addition, cocaine reward and self-administration are partly promoted by SIRT1 dependent activation of ERK in the MAPK pathway (Y. Li, Xu, McBurney, & Longo, 2008; Renthal et al., 2009). Both sirtuins and MAPK are highly conserved signaling molecules with pleiotropic roles (E. K. Kim & Choi, 2010; Vassilopoulos, Fritz, Petersen, & Gius, 2011). Here, we aim to study the interplay among sirtuin SIRT1, ERK1/2, and the nicotine-induced microRNA miR-80 in mediating nicotine-induced early onset diseases.

To give a small background on our genes of interest, sir-2.1/SIRT1 belongs to the sirtuin family of proteins and is an NAD+-dependent protein deacetylase that is involved in transcriptional repression, stress response, lifespan (Bamps, Wirtz, Savory, Lake, & Hope, 2009), and apoptosis (Greiss, Hall, Ahmed, & Gartner, 2008). Meanwhile, mpk-1/ERK1/2 is the downstream effector of the MAPK pathway and is considered a central hub in *C. elegans* development, cell proliferation and differentiation, apoptosis, stress response (Sundaram, 2006), and lifespan (Okuyama et al., 2010). In addition, miR-80 belongs to the miR-58 family in *C. elegans* which is highly abundant and expressed at all developmental stages (Alvarez-Saavedra & Horvitz, 2010). Interestingly, members of the miR-58 family are orthologous to the bantam miRNA in Drosophila that controls cell proliferation and apoptosis (Brennecke, Hipfner, Stark, Russell, & Cohen, 2003). miR-80 mutants are long lived. Its loss of function prevented the age-dependent decline of pharyngeal pumping and age-related pigments, decreased fecundity, and slenderized worm body (Vora et al., 2013). This suggests that its presence is pro-ageing. Though

single miR-80 mutants did not have an abnormal phenotype, it rescued the sluggish, locomotory, as well as egg laying defect in miR-58 family multimutant (Alvarez-Saavedra & Horvitz, 2010). Based on our study, miR-80 was the only miRNA to be upregulated in response to both low and high nicotine doses (F. A. Taki et al., 2014; F. A. Taki et al., 2013). Based on benefits dependent on miR-80 deletion, we propose that its increased expression might contribute to physiological complications in response to nicotine.

Material and methods

Strains from CGC

N2 (lab WT strain), ga111 (ts and *lof* mpk-1), JK3929 (ga117), MT2124 (let-60 n1046), MT13949 (mir-80 (nDf53 III)), MT15563 (nDf53 III; mir-58 (n4640) IV; nDf54 X), FX863 (acr-7(tm863 II), RM509 (ric-3(md158 IV), VC199 (sir-2.1(ok434 IV), JR667 (unc-119(e2498::Tc1 III; wIs51 V), GR1366 (mgIs42 [tph-1::GFP + rol-6(su1006)]).

Worm maintenance and treatment

Worms were maintained on solid NGM (0.3% NaCl, 0.25% peptone, 1.7% agar, 0.5% cholesterol, 0.1% of 1M MgSO4, 0.1% of 1M CaCl2, and 2.5% of 1M KPO4) at 20°C. For treatment, we used two nicotine doses based on our previous studies (F. Taki et al., 2013; F. A. Taki et al., 2014; F. A. Taki et al., 2013; F. A. Taki & Zhang, 2013). Treatment plates as previously described (F. A. Taki et al., 2014) such that the final nicotine dose for the low and high treatment groups were 20μM and 20mM, respectively.

Embryos were collected via the bleaching method before treatment ((70% water, 10% NaOH, 20% bleach) (Stiernagle, 2006). Once hatched and paused at L1, worms were transferred

onto control and treatment plates that were seeded with the food source OP50 15-30 minutes prior to the experiment. The treatment period lasted for 30 hours only at 20°C unless otherwise noted (e.g. temperature sensitive worms grown at 22°C and 25°C were exposed for 28 and 22 hours, respectively).

Strains made

Ex myo-2 mpk-1 GFP was expressed in N2, miR-80 (nDF53), and sir-2.1 (ok434) backgrounds. IBI high speed mini plasmid kit was used for plasmid mini-preps. Plasmids pPD95_79 and L2531 were purchased from Addgene, PFU polymerase (MBL international) was used for all steps of construct construction; Platinum Taq polymerase (Invitrogen/Lifetechnologies) was used for screening; NheI and BglII (NEB) were used for sequential double digestion. All ligation reactions were performed overnight at 4 °C using NEB ligase (5µl reaction of 3.5µl fusion, 0.5µl TA vector, 0.5µl enzyme, 0.5µl buffer) (Supplementary figure 3.1).

Plasmid construction

GFP was amplified from pPD95-79 using forward and reverse primers 5'-AATAATGGAGGGCAGAATCCTGTTATTGGCCAAAGGACC-3'and 5'-GAAGATCTTCAAGTTGGTAATGGTAGCGACCG-3', respectively. mpk-1a cDNA (gift from Lee lab) was amplified using the following forward and reverse primers 5'-CTAGCTAGCTAGATGGCCGACGGAGAAGCGGTTATC-3', and 5'-GGTCCTTTGGCCAATAACAGGATTCTGCCCTCCATTATT-3', respectively. PCR products were purified by spin column purification and then used for fusion PCR. The latter was performed using forward and reverse primers 5'-

CTAGCTAGCTAGATGGCCGACGGAGAAGCGGTTATC-3', and 5'-GAAGATCTTCAAGTTGGTAATGGTAGCGACCG-3', respectively and was spin column-purified before further steps. dA overhangs were added by incubating the fusion with taq polymerase reaction without primers for 30 minutes at 70°C. The fusion with sticky dA ends was then ligated to a pGXT vector (modified pGMET vector was a gift from the Zhu lab) and transformed into 50µl competent cells. Positive colonies were screened by colony PCR and sequential digestion with M-13-F and R primers, respectively.

Both TA-fusion and L2531 plasmids (Addgene) were double digested with NheI at 37°C overnight. The digested product was spin-column purified and used for the BglII digestion at 37 °C overnight following manufacturer's protocol. Digested products were separated on 1% gel. Fragments with the correct sizes were excised at UV=365 nm in less than 30 sec and were used for the overnight ligation at 4°C. Ligated product (L2531-Fusion) was then transformed into DH5α E. coli competent cells, prepared according to published protocol (D. A. Wright et al., 2006). 5µl of ligation solution was added to thawed cells and were left from 30 minutes on ice before being heat shocked for 90 seconds in a 42°C water bath. Cells were allowed to recover for 2 minutes on ice before adding 300µl LB and were left for 1 hour on a shaker at 37°C. Then cells were spread on LB plates infused with 100ug/ml ampicillin and were left to grow overnight at 37°C. 10 colonies were then grown and were used for plasmid isolation. Plasmids were then screened using forward and reverse primers 5'-ATTGGCCAAAGGACCCAAAGG-3' and 5'-TACGAATGCTGAGCGCTG-3'. Positive colonies were sequentially digested using NheI and BglII overnight at 37°C. The digested fragment was assessed on a gel relative to a DNA ladder. Positive plasmids were then Sanger sequenced for further validation before injecting to worm gonads. After confirmation, one plasmid was injected into gonads of at least 10 of each of the

three worm strains: N2, miR-80 (nDf53), and sir-2.1 (ok434) worms. Positive worms with pharyngeal GFP expression were spotted using a fluorescence stereoscope. Worms were then transferred to individual plates and were grown for at least 8 generations before the experiment.

Seam cell count

GFP fluorescent seam cells were used as a marker to confirm the developmental stage of worms treated with nicotine relative to the no-treatment control. JR667 (unc-119(e2498::Tc1) III; wIs51 V) strain was used to visualize and count the number of seam cell in response to nicotine. Worms were treated for 30 hours until the L3/L4 molting stage. Worms were collected in an Eppendorf tube and then mounted on 2% agarose pads. Worms were paralyzed with 5mM levamisole, allowed to dry, and were then covered with a coverslip. Worms were then visualized using Zeiss inverted fluorescent microscope at 10X magnification. Pictures were taken, and the numbers of GFP fluorescent seam cells were counted per worm side (Figures 3.3BC; Table 3.1).

Length measurements

After worms were exposed to nicotine for 30 hours, worms were washed off the plate and mounted on 2% agarose slide. Worms were paralyzed with 5mM levamisole, dried, and covered with a coverslip. Worm images were taken using a camera mounted to Zeiss inverted microscope at 10X magnification. Length was measured for at least 30 worms per treatment group using imageJ software. One-way ANOVA was used to compare length measurements among treatment groups. Post-Hoc test was applied for pairwise comparisons and Bonferroni correction was applied. Statistical significance was based on $p<0.05$.

Pharyngeal pumping assay

Worms were transferred into a 12 well NGM plate with or without nicotine. Plates were previously seeded with 10µl of OP50 in the center. Worms were left to acclimate for two to three

hours before assessing their pumping rate. A minimum of 30 second -videos were taken per worm. At least 15 worms were tested per treatment group. One to three trials were performed per strain. Each trial was replicated three times. Videos were then played back at a slower speed to allow for manual pharyngeal counting to compute the pharyngeal pumps per minute for each worm. Data from all worms were pooled for statistical analyses. Residuals for raw data were checked for normality. Based on the latter, log transformation was done and was used as input for ANOVA in SPSS(20). Bonferroni correction was applied and statistical significance was based on $p<0.05$.

Heat maps

Mev was used to construct heat maps. Unsupervised hierarchical clustering was based on optimized treatment leaf order, Euclidean distance, and average linkage clustering (Figures 3.3B; 3.4B).

Results

The relationship between miR-80, MAPK, and sir-2.1

As previously mentioned, fos-1 and cmk-1 were transgenerationally enriched in response to parental nicotine exposure based on our predicted miRNA network (F. A. Taki et al., 2014). Previous studies showed that both factors promote substance use disorders via sirtuin and miRNA-dependent mechanisms (Fusco et al., 2012; Robison & Nestler, 2011). One of the members of the aforementioned miRNA network was miR-80. It was upregulated in both parent (F0) generation and grand-offspring (F2) generation worms (F. A. Taki et al., 2014; F. A. Taki et al., 2013). Based on our KEGG analysis, MAPK pathway was enriched for miR-80 predicted

targets (Figure 3.1A; 3.2AC). This pathway is commonly and consistently implicated in nicotine-induced disorders and responses (Figure 3.1A) (C. Y. Li et al., 2008) and was recently reported to be downstream of SIRT1(Y. Li et al., 2008; Renthal et al., 2009) (Figure 3.1A). Using the tissue-specific expression tool developed by Chikina et al. (Chikina, Huttenhower, Murphy, & Troyanskaya, 2009), we show the fos-1, cmk-1, sir-2.1, and mpk-1 are predicted to be co-expressed in most tissues in the worm (Figure 3.1B) and could potentially work together in response to nicotine. Our analysis showed that miR-80 was the only transgenerational miRNA predicted to co-target both sir-2.1 and mpk-1 in the worm (Figures 3.2ABC). Therefore, in this study, we plan to investigate the interplay between miR-80, mpk-1, and sir-2.1 in response to post-embryonic nicotine exposure.

Post-embryonic nicotine exposure negatively impacted growth and development

L1 worms were transferred onto NGM plates with or without nicotine for a 30 hour exposure period. Two nicotine doses 20µM and 20mM were used based on our previous behavioral and genetic studies. After the period passed, we noticed that worms exposed to the high nicotine dose were smaller than both control and low treatment groups (Figure 3.3A). To accurately identify the developmental stage, we used the seam cell count as a biomarker for development. Seam cells are found laterally on both sides along the worm body. During the first three larval stages, the seam cells go through a stem cell-like division pattern. In L1, there are 10 seam cells per worm side (H0, H1, H2, V1–6 and T). Before each molt, three events reiterate starting with, in most cases, asymmetric cell division, hypodermal fusion or cell differentiation, and reestablishment of contact between seam cells. The last cell division occurs at the L3/L4 molt after which 16 seams cells terminally differentiate in the L4 stage per worm side, fuse in adults to form a syncytium and give rise to the cuticle (Z.F. Altun & D.H. Hall, 2009). So, we

used the strain JR667 of which seam cells express GFP to facilitate counting seam cells in the worm and the inference of the developmental stage.

Our results showed that about 50% of the control worms had around 28 cells per side. This number slightly decreased to 25 when worms were treated with the low nicotine dose. However, about 50% of the worms treated with the high dose only had up to 11 seam cells per side. As shown in the heat map (Figure 3.3B), the highest percentage of worms (14%) had 30 seam cells per side in control, while the highest percentage of worms (20%) had 17 seam cells in response to the low nicotine dose. Meanwhile, the greatest majority of worms (36%) had 10 seam cells after 30 hours of exposure to 20mM nicotine (Figure 3.3BC). The worms should range between late L3 to early L4 30 hours post-hatching. Typically, L4 worms have exactly 16 unfused seam cells per side (Z.F. Altun & D.H. Hall, 2009). Our results showed that about 12% of control worms had 16 seams cells per side, while 15% of the worms treated with the low nicotine dose had this number. As for the high treatment group, only 4% of the worms had 16 seam cells per side. This data shows that the high nicotine dose delayed development of the worm population such that worms were arrested at the L1 stage [$F(2,134)=93.55$; $p<0.001$]. On the other hand, worms exposed to the low nicotine dose had fewer seam cells than control and may have finished the proliferative state concomitant with the molting stage (Monsalve, Van Buskirk, & Frand, 2011). This suggests that more control worms were still in the L3/L4 molting stage, while more worms progressed to early L4 in response to the low nicotine treatment (Figure 3.3BC).

Nicotine altered worm length

We measured the worm length after 30 hours of nicotine exposure using imageJ software. Chronic early nicotine exposure significantly impacted worm length [$F(2,141)=723.40$; $p<0.001$]. Pairwise comparisons revealed that this was mainly due to the high nicotine dose which resulted in a 46% decrease in the worm length ($p<0.001$) (Figure 3.4A).

We were interested in testing the effect of 30 hour nicotine exposure in different genetic backgrounds. In an mpk-1 sensitive background (ga111 grown at 20°C), the effect remained significant [$F(2,100)=641.24$; $p<0.001$]. This effect became stronger when mpk-1 was partially inactivated (ga111 grown at 22°C) [$F(2,69)=1160.67$; $p<0.001$] and completely inactivated (ga111 grown at 25°C) [$F(2,92)=755.73$; $p<0.001$]. This effect was similar when nicotine was exposed to miR-80 and sir-2.1 loss of function mutants [$F(2,92)=643.40$; $p<0.001$] and [$F(2,96)=646.61$; $p<0.001$], respectively. On the other hand, when MAPK was overactivated as in let-60 (n1046), the impact of early nicotine exposure was less severe [$F(2,89)=386.65$; $p=0.001$]. So, the high nicotine dose was associated with a significant decrease in the worm length in all the mutant backgrounds. The least decrease was in the miR-80 (nDf53) background (43%), followed by sir-2.1 (ok434) (45%), let-60 (n1046) (46%), ga111 at 25°C (47%), ga111 at 20°C (51%), and ga111 at 22°C (59%) at $p<0.001$. Interestingly, the low nicotine dose increased the worm length in only ga111 background. The length increased by 8% when ga111 was grown at the permissive temperature 20°C ($p<0.001$) and by 6% at the restrictive temperature 25°C that results in complete deactivation of mpk-1 (Figure 3.4A). As a summary, the high dose treatment had similar impacts in almost all the tested genetic backgrounds and they all clustered together. This means regardless of the increase in length in their control and low counterparts relative to the N2 background, the high dose inhibited the worm length (Figure 3.4A).

Since the weakest impact for nicotine exposure on worm length was in the let-60 (n1046) background with an overactivated MAPK pathway, we wondered if nicotine-induced mpk-1 in the pharynx would result in a similar effect on length. So, we generated three strains where mpk-1 GFP fusion was overexpressed in the worm's pharynx using the myo-2 promoter as described in the methods section. This construct was expressed in N2 worms and two loss of function mutants: miR-80 (nDf53), and sir-2.1 (ok434). We refer to these strains as N2Ex, miR-80Ex, and sir-2.1Ex. GFP fluorescent pharynxes were used as a marker to pick the worms of interest using a fluorescent stereoscope. As expected, the severity of nicotine-induced reduction in worm length was lower than N2, miR-80 (nDf53), and sir-2.1 (ok434) without pharyngeal mpk-1 overexpression. The F-score in response to nicotine treatment dropped to [$F(2,93)=89.01$; $p<0.001$] in N2Ex, [$F(2,108)=63.06$; $p<0.001$] in miR-80Ex, and [$F(2,109)=134.73$; $p<0.001$] in sir-2.1Ex. In particular, the high nicotine dose decreased the length by 42%, 43%, and 50% ($p<0.001$) in N2Ex, miR-80Ex, and sir-2.1Ex, respectively (Figure 3.4A).

When comparing the effect of each treatment per strain relative to the N2 background (e.g. each high group per strain was subtracted from the high group of N2), only let-60 (n1046), miR-80 (nDf53), and sir-2.1 (ok434) had a proportional increase in length in the control, low, and high groups (6-11%). However, the major difference was in the ga111 background when grown at 20, 22, and 25°C. The increase was no longer proportional when comparing control and low groups with the high groups. Worms in the high treatment group increased by only 6-10% relative to their N2 counterparts, while the controls and lows increased by 19-45% relative to N2. Interestingly, when mpk-1 was overactivated in miR-80Ex and sir-2.1Ex, neither the low nor the high groups were proportional to their controls. The low dose decreased the length by 9-10%, while the high dose decreased the length by 2-4% (Figure 3.4B).

We took notice of the length patterns in three backgrounds: N2, miR-80 (nDf53), and sir-2.1 (ok434) and focused on the impact of pharyngeal mpk-1 overexpression per treatment group. The first set of comparisons is shown in column (A) and the second set is shown in column (B). The top row (blue) is comparing control groups, the middle row (red) is comparing low treatment groups, and the bottom row (green) is comparing high treatment groups in different backgrounds, respectively. In column (A), we checked how pharyngeal overexpression of mpk-1 shifted the length per genetic background. We noticed that pharyngeal overexpression of mpk-1 decreased length in N2 ($p=0.005$) and miR-80 (nDf53) (0.001) backgrounds by 11%. The low dose didn't further decrease the length in N2 background (11%, $p=0.001$) while it decreased the length by 18% from miR-80 to miR-80Ex ($p<0.001$). The high dose did not shift the length further after mpk-1 overexpression in the pharynx in neither N2, nor miR-80 (nDf53) backgrounds. Interestingly, pharyngeal overexpression of mpk-1 in sir-2.1 (ok434) did not impact length in the control groups; however, both the low and high nicotine doses significantly decreased the length from sir-2.1 to sir-2.1Ex by 15% ($p<0.001$) and 17% ($p=0.014$), respectively (Figure 3.4C). This suggests that (i) mpk-1 is inversely proportional with length, (ii) mpk-1 is downstream of miR-80 and sir-2.1, (iii) the low dose further increased mpk-1 activation, which in turn decreased the length, (iv) the high dose did not further increase mpk-1 activation in N2 and miR-80 (nDf53) backgrounds, and (v) sir-2.1 (*lof*) mutation had lower mpk-1 activation which increased upon low and high nicotine exposure and consequently decreased length.

In column (B), we looked into the impact of miR-80 (nDf53) and sir-2.1 (ok434) on length as a function of pharyngeal mpk-1 overexpression and nicotine exposure. In the absence of nicotine (i.e. controls, first row), miR-80 loss of function increased the length by 10% relative to N2 ($p=0.017$) and this effect was lost when mpk-1 was overexpressed in the pharynx in miR-

80Ex. Interestingly, sir-2.1Ex length remained 13% higher than N2Ex (p=0.003). This is consistent with the previous propositions where sir-2.1 loss of function had lower levels of endogenous mpk-1. The low nicotine dose did not alter the increase in length in miR-80 (nDf53) background (11%, p=0.003); however, the pattern shifted once mpk-1 was overexpressed in the pharynx such that it reversed and decreased by 10% (p=0.01). In addition, sir-2.1 (ok434) loss of function could no longer increase the length in the presence of exogenous pharyngeal mpk-1. Finally, similar patterns were observed in the high treatment groups though they were not statistically significant suggesting that the high nicotine activates mpk-1 to a high level such that the inhibitory effect on length finally plateaus and is no longer impacted by further activation of exogenous mpk-1, even in sir-2.1 (ok434) loss of function background (Figure 3.4C). This suggests that (i) miR-80 and sir-2.1 are inversely proportional with length (ii) miR-80 and sir-2.1 have a positive relation with mpk-1 (iii) mpk-1 contributes to the 10% increase in length in miR-80 (nDf53) background, and (iv) the low and high nicotine doses activate mpk-1 and impact worm length even in miR-80 and sir-2.1 loss of function backgrounds.

Is nicotine-induced developmental delay a consequence of pharyngeal pumping inhibition?

High doses of nicotine have been shown to cause muscular paralysis through over-activation of neuromotor signaling pathways (Matta et al., 2007). *C. elegans* is a filter feeder that depends on autonomous rythmic pharyngeal pumping action to swallow and ingest their food. This pumping action depends on neuronal input to function efficiently (Avery & You, 2012) and is affected by the mechanochemical signals associated with the presence of food (Bargmann, 2006; Goodman, 2006). Taken collectively, we hypothesized that the observed developmental delay is a side-effect of nicotine-induced inhibition of pharyngeal pumping. Perhaps pumping inhibition is severe and results in worm starvation which consequently results in a delayed

development. To test this hypothesis, we measured the pharyngeal pumping rate in response to the low and high nicotine doses and compared the rate to their untreated control as described in the methods section.

Our results showed that nicotine dramatically inhibited pharyngeal pumping [$F(3,145)=33.77$; $p<0.001$]. This significant effect was mainly due to the high nicotine dose that decreased pumping by 67% ($p<0.001$). To determine if this decrease was due to developmental delay rather than a direct effect of nicotine exposure, we compared the pharyngeal pumping with size matched larvae that were not exposed to nicotine. Based on our seam cell count and mean length measurements, we deduced that most of the worms treated with the high nicotine dose are similar to L1 larvae. Worms were synchronized and left to hatch for about 18 hours as described in the methods section. Hatched L1 were plated onto NGM seeded with OP50 and left to acclimate and adapt for up to three hours before the pumping assay. The pumping rate for L1 control was 68% higher than the high treatment group ($p<0.001$) (Figure 3.5A). This suggests that the decrease in the pumping rate is a direct effect of nicotine rather than a side-effect of developmental delay. We also exposed the worms to nicotine in the absence of food. Even though all worms including control and low groups arrested in development, the same pumping inhibition trend was observed and was proportional to the nicotine dose. Due to the low pumping rate in the absence of food, the overall effect was not statistically significant [$F(2,74)=1.48$; $p=0.234$] (Figure 3.5A).

We were intrigued to identify some of the molecular contributors of nicotine-induced pumping inhibition. We decided to examine the effect of nicotine on pharyngeal pumping in worms of which MAPK pathway is sensitive or altered. mpk-1 (ga111) is a temperature sensitive strain such that mpk-1 is inactivated with increasing temperatures (i.e., worms are sterile at 25°C

due to complete inactivation of mpk-1). As shown in Figure 3.5A, nicotine inhibited the pumping rate in a dose dependent manner in mpk-1 (ga111) grown at 20°C [$F(2,42)=17.50$; $p<0.001$]. Pairwise comparisons revealed that the low dose was sufficient to decrease the pumping rate by 36% ($p=0.008$), while the high dose decreased the pumping rate by 77% ($p<0.001$). The effect remained evident when mpk-1 (ga111) worms were grown at 25°C [$F(2,31)=10.89$; $p<0.001$], but that was only due to the high nicotine dose of which rate decreased by only 42% ($p=0.003$). Interestingly, when this strain was grown at 22°C, the effect of nicotine on pharyngeal pumping was no longer evident [$F(2,44)=0.39$; $p=0.678$]. The same recovery was observed in mpk-1(ga117) [$F(2,33)=1.441$; $p=0.251$] where mpk-1 is reported to be completely inactivated (Lackner & Kim, 1998). Then we wondered what would happen if we exposed nicotine to worms with overactivated MAPK pathway. For that, we used let-60 (n1046) strain and noticed that nicotine did impact the pumping rate [$F(2,42)=15.50$; $p<0.001$]. Interestingly, the low nicotine dose slightly increased the pumping rate by 34% ($p=0.046$) while the high dose consistently decreased the pumping rate by only 42% ($p=0.009$) (Figure 3.5A).

We repeated the experiment by exposing worms with a loss of function mutation in miR-80 (nDf53) with nicotine. Our results showed that nicotine could no longer significantly impact pumping rate in this genetic background [$F(2,42)=1.77$; $p=0.183$]. miR-80 is a member of the miR-58 miRNA family that is highly abundant and expressed throughout development in *C. elegans*. This family is orthologous to *bantam* miRNA in *Drosophila* which is involved in cell proliferation and apoptosis (Alvarez-Saavedra & Horvitz, 2010). The rescue phenotype was not as evident, but was also observed after nicotine was exposed to worms lacking the entire miR-58 family [$F(2,42)=3.59$; $p=0.036$] where neither low nor high doses differed significantly from

control. In addition, the recovery of the nicotine-induced pumping inhibition was observed in worms with loss of function mutation in sir-2.1 (ok434) [$F(2,86)=4.104$; $p=0.02$] (Figure 3.5A) .

Nicotine was reported to delay development of skeletal muscle growth in cell lines via nAChR-α7 signaling (Kawakita A et al., 2008) along with secondary spinal motor neurons (Svoboda et al., 2002). To test the role of nAChR- α7 in *C. elegans*, we found the pumping rate was only slightly impacted, but was no longer severely impacted in worms with dysfunctional acr-7 (tm863) after nicotine exposure [$F(2,42)=3.38$; $p=0.043$] which is consistent with previous reports using a much lower (1mM) nicotine dose (Saur et al., 2013). Interestingly, worms which lost the function of ric-3 (md158) still showed a severe inhibition of pharyngeal pumping [$F(2,42)=11.062$; $p<0.001$] and especially in response to the high nicotine dose which resulted in a 77% decrease in the pumping rate ($p<0.001$). ric-3 is responsible for the maturation and function of a number of nAchRs and was previously reported to mediate resistance against nicotine-induced paralysis (Halevi et al., 2002). A possible reason for the discrepancy is the much lower concentration used in their study (100µM) relative to our high dose (20mM) (Figure 3.5A).

Taken collectively, our data failed to show a strong correlation between nicotine-induced developmental delay and size retardation with inhibited pharyngeal pumping. In other words, the smaller size and delayed development could be dissociated from the reduced pumping rate. For example, mpk-1 (ga111) grown at 20°C exposed to the low nicotine dose had a lower pumping rate yet was larger in length than the control. In addition, mpk-1 (ga111) grown at 22°C was resistant to nicotine-induced pharyngeal paralysis yet the worms exposed to the high nicotine dose resulted in the most severe worm shortening. More so, let-60 (n1046) worms exposed to the low nicotine dose had increased pumping rate yet were similar in size to their control worms.

Lastly, miR-80 (nDf53) and sir-2.1 (ok434) still showed great size retardation despite having an improved pumping rate relative to their control. It is noteworthy that ga117 control had significantly lower pumping rate than the N2 control ($p<0.001$) (Figure 3.5A).

The effect of nicotine in the presence of mk-1 inhibitor (U0126)

As mentioned earlier, both mpk-1 and sir-2.1 are predicted targets for miR-80. We were interested in testing a possible functional feedback among them in response to early nicotine exposure. So, U0126 was diluted in DMSO solvent and was mixed with the worm OP50 food source after which they were spread on the media and left to dry for 1 hour. The final concentration for U0126 was 50µM based on previous reports (F. Chen, Mackerell, Luo, & Shapiro, 2008). Synchronized L1 worms were transferred to NGM plates seeded with OP50 and DMSO, U0126, or U0126 on 20mM nicotine (high dose) NGM plates. Worms were left to for 30 hours on treatment before the pharyngeal pumping rate was assessed. Our results showed that U0126 partially negated the effect of nicotine on pharyngeal pumping in N2 worms [$F(2,110)=12.06$; $p<0.001$]. The pharyngeal pumping was only reduced by 25% ($p=0.015$) instead of the 67% reduction in response to the high nicotine dose in N2 worms. Interestingly, miR-80and sir-2.1 loss of function mutations could no longer rescue nicotine-induced pumping inhibition in the presence of U0126 with overall impacts of [$F(2,96)=37.03$; $p<0.001$] and [$F(2,86)=31.13$; $p<0.001$], respectively. This combination treatment decreased the pumping rate by 32% ($p<0.001$) in miR-80 (*lof)* and by 49% ($p<0.001$) in sir-2.1 (*lof*) backgrounds (Figure 3.5B).

The impact of pharynx-specific overexpression of mpk-1on nicotine-induced responses was context dependent

Since partial inhibition of mpk-1 by U0126 alleviated nicotine's inhibitory effect on pharyngeal pumping, we deduced that nicotine-induced pumping inhibition could be partly due to the overactivation of mpk-1. We planned to validate this hypothesis by testing the effect of nicotine in a hyperactivated mpk-1 background. For that, we generated three strains where mpk-1 GFP fusion was overexpressed in the worm's pharynx using the myo-2 promoter as described in the methods section. This construct was expressed in N2 worms and two loss of function mutants: miR-80 (nDf53), and sir-2.1 (ok434). We refer to these strains as N2Ex, miR-80Ex, and sir-2.1Ex. GFP fluorescent pharynxes were used as a marker to pick the worms of interest using a fluorescent stereoscope. Nicotine treatment altered the pumping rate in N2Ex [$F(2,131)=24.96$; $p<0.001$], miR-80Ex [$F(2,137)=43.25$; $p<0.001$], and in sir-2.1Ex [$F(2,87)=6.44$; $p=0.002$]. In all strains, the pumping inhibition was due to the high nicotine dose while the low dose had no effect. Similarly to the N2 background, the high nicotine dose reduced the pumping rate by 68% ($p<0.001$). On the contrary, the high nicotine dose reduced the pumping rate by 46% in miR-80Ex strains despite having no effect in miR-80 (*lof*) mutants without mpk-1 overexpression in the pharynx. Similarly, sir-2.1 (*lof*) worms were no longer resistant to nicotine's inhibitory effect when mpk-1 was overexpressed in the pharynx such that the pumping rate decreased by 50% ($p=0.002$) (Figure 3.5C). As a summary, our results suggest that (i) the high nicotine dose inhibits pharyngeal pumping through the overactivation of pharyngeal mpk-1, miR-80, and sir-2.1, (ii) mpk-1 works downstream of miR-80 and sir-2.1, and (iii) that miR-80 and sir-2.1 are positively correlated with mpk-1.

Discussion

In this study, we aimed to understand the role of miR-80 in response to direct nicotine in *C. elegans* larvae. Worms were exposed to two nicotine doses during their post-embryonic

development. This resulted in two noticeable effects: inhibited development and reduced pharyngeal pumping. We showed that mpk-1, sir-2.1 and miR-80 contribute to the pharyngeal pumping phenotype and that the latter can be dissociated from the developmental delay.

MS80: mpk-1, sir-2.1, and miR-80

Our previous expression and bioinformatics analyses highlighted cmk-1 and fos-1 (fosbZip transcription factor family) as transcripts downstream of nicotine-responsive microRNAs like miR-80 (Figure 3.1). cmk-1, predicted to be a direct target of miR-80, activates crh-1/CREB (cyclic AMP-responsive element-binding protein) transcription (Kimura et al., 2002). crh-1/CREB promote long term memory (Lakhina et al., 2015; Sugi, Ohtani, Kumiya, Igarashi, & Shirakawa, 2014), cell survival and proliferation, apoptosis, differentiation, metabolism, and immune response (Mayr & Montminy, 2001). crh-1/CREB binds promoters and recruit co-activators or co-repressors to genes responsive to drugs of abuse like cocaine amphetamine, opiates (Robison & Nestler, 2011), and nicotine (Brunzell, Mineur, Neve, & Picciotto, 2009). CBP is a histone acetyltransferase CBP (CREB-binding protein) that is a co-activator of CREB (Kwok et al., 1994). The histone acetylase activity of cbp-1 and crh-1 dependent transcriptional activation was perturbed in a *C. elegans* neurodegenerative model (Bates, Victor, Jones, Shi, & Hart, 2006). Interestingly, cbp-1 was repressed by miR-80 in *C. elegans*, and the expression levels of both were reversed with dietary restriction (Vora et al., 2013). Meanwhile, sir-2.1/SIRT1 was predicted as a target for miR-80 and our data brought to light a potentially positive relationship between them. Contrary to cbp-1/CBP, sir-2.1/SIRT1 is a histone deacetylase that can serve as a corepressor (Xie et al., 2009) as well as an activator (Fusco et al., 2012; Gao et al., 2010) of and for CREB. On the other hand, miR-80 is upregulated

in response to nicotine and this suggests that it might induce crh-1/CREB mediated gene regulation through sir-2.1/SIRT1.

sir-2.1/SIRT1 sirtuins are conserved class III histone deacetylases that not only impact the chromatin states, but are also members of transcriptional repressive complexes and have also been shown to result in posttranslational modifications of non-histone proteins like transcription factors (Robison & Nestler, 2011). SIRT1 is crucial for the orchestration of transcriptional programs relative to the stress and energy states in an organism (Noriega et al., 2011) and have been shown to have roles in neurodegenerative diseases, oxidative and environmental stress response, caloric restriction, ageing, lipid metabolism, DNA damage response, apoptosis, cell survival, and cancers (Rahman & Islam, 2011). Recently, SIRT1 was shown to promote reward and self-administration in response to cocaine and morphine (Ferguson et al., 2013; Ferguson et al., 2015; Renthal et al., 2009) and this response involved ERK1/2 activation downstream of SIRT1 (Y. Li et al., 2008; Renthal et al., 2009). Since then, SIRT1 and MAPK were shown to have a synergic relationship in promoting neuronal apoptosis after TBI (Zhao et al., 2012) and were positively correlated in response to oxidative stress in ischemic-reperfused cardiomyocytes (Becatti et al., 2012) and in vitiligo skin (Becatti et al., 2014). This positive relationship was also deduced from our data using mpk-1/ERK2 inhibitor U0126 and mpk-1/ERK2 overexpression in the pharynx.

mpk-1/ERK1/2 is a part of the mitogen-activated protein kinase (MAPK) pathway. The latter are evolutionarily conserved kinases that relay extracellular signals and affects cellular processes such as growth, proliferation, differentiation, migration and apoptosis (Cargnello & Roux, 2011; Dhillon, Hagan, Rath, & Kolch, 2007). Given their pleiotropic roles, MAPK signaling is implicated in many diseases like cancers, and neurodegenerative diseases like AD,

PD, and ALS (E. K. Kim & Choi, 2010). It is also enriched for drugs of abuse and particularly nicotine (C. Y. Li et al., 2008). Interestingly, MAPK was enriched for miR-80 regulated pathways (Figure 3.1A) and mpk-1/ERK2 was predicted to interact with miR-80 (Figure 3.2). Therefore, this study is the first to investigate the interplay among miR-80, MAPK, and sirtuin signaling in response to nicotine.

Developmental arrest

Response to stress and developmental arrest is systematic and involves cell non-autonomous signaling. The most studied cases of *C. elegans* developmental L1 arrest is in response to starvation is recognized as L1 quiescence (Baugh, 2013). Our results showed that the exposure to the high nicotine dose was associated with an L1-arrest like state (Figure 3.3). Was nicotine a direct cause for this phenotype? Alternatively, was this developmental arrest due to nicotine-induced starvation as a result of pharyngeal pumping inhibition? We argue that the phenotype is only partially mediated by nicotine-induced altered feeding rate; however, we were able to dissociate worm growth from pharyngeal pumping rate. This suggests that there is also a direct effect of nicotine on worm growth and this mechanism remains unknown. For example, ga117 (mpk-1 *lof*), ga111 grown at 22°C, miR-80 (*lof*), sir-2.1 (*lof*) were resistant to nicotine's effect on pharyngeal pumping (Figure 3.5A), but those worms were still arrested suggesting that other factors are involved. Candidate factors include insulin-signaling factor daf-16 and miR-235 of which loss of function mutants are defective in starvation induced arrest. Starvation activates daf-16 which upregulates miR-235 in the worm. miR-235 was actually downregulated in response to our nicotine exposure (F. A. Taki et al., 2013) and miR-235 (*lof*) mutants did not rescue nicotine-induced developmental arrest (data not shown). Also, if left on nicotine, some of the worms do grow though at a much slower pace and remain smaller in size and can still

produce much fewer eggs (data not shown). This does not happen in starving worms that remain arrested. Another support is that miR-80 (nDf53) (*lof*) mutants had a decreased survival rate in response to starvation-induced L1 arrest (X. Zhang, Zabinsky, Teng, Cui, & Han, 2011). On the contrary, miR-80 (nDf53) worms were resistant to nicotine's effect on pumping which suggests that nicotine's impact on growth is not a side-effect of starvation. miR-80 (*lof*) was associated with a dietary restriction (DR)-like global metabolism (Vora et al., 2013). So, if miR-80 loss of function mirrors caloric restriction in the worm, then we would expect the opposite with miR-80 gain of function as was observed after nicotine treatment (F. A. Taki et al., 2014; F. A. Taki et al., 2013). Recently, de Lucas et al. showed that miR-58 family, which included miR-80, interacts with TGFβ signaling pathway to regulate worm growth and stress resistance (de Lucas, Saez, & Lozano, 2015); however, disruption of TGFβ is linked to dauer phenotypes and did not affect the survival of starvation-arrested L1 worms (Baugh, 2013). Taken together, we conclude that there is a direct effect of nicotine on worm's development which can be dissociated from its effect on pharyngeal pumping and that the mechanisms behind this effect remain elusive.

The roles of sir-2.1 and miR-80 in nicotine induced pharyngeal pumping inhibition

Only the high nicotine dose inhibited pharyngeal pumping in N2 background (Figure 3.3A). This effect was rescued in miR-80 (*lof*) and sir-2.1 (*lof*), and was partially rescued in acr-7 (*lof*) and ric-3 (*lof*). miR-80 (*lof*) was associated with better pumping at old age (Day 11) relative to N2 worms (Vora et al., 2013) and this provides support for miR-80's role in improving pumping in different conditions (e.g. nicotine, ageing). Does this recovery involve an upregulation of MAPK?

Nicotine induced pumping inhibition in different mpk-1 backgrounds

Nicotine has been shown to cause hypercontraction in dissected pharynxes through MC motor neuron neurotransmission which activates downstream nAChR eat-2 with eat-18 (McKay, Raizen, Gottschalk, Schafer, & Avery, 2004). Nicotine causes pharyngeal tetanus defined as contractions lasting more than 30 seconds as opposed to less than 1 second in normal pumps (Avery & Horvitz, 1990). We treated worms of different mpk-1 backgrounds and checked their pumping rates as discussed below.

ga111 is a weak loss of function allele that affects MEK binding site and its consequential phosphorylation of mpk-1 and its activation. This mutation is associated with a germ line sterility phenotype at higher temperatures which are restrictive for mpk-1 activity. ga111 worms are superficially wild type when grown at 20°C (Lackner & Kim, 1998) and might be partially non-functional as it could rescue the IR-induced apoptosis phenotypes in let-60 (*gof*) and lip-1 (*lof*) worms (Rutkowski et al., 2011). However, ga111 remain sensitive to stimuli that activate mpk-1. Though at a lower rate, IR was still able to activate MAPK in ga111 worms grown at 25°C (Rutkowski et al., 2011). This means that mpk-1 (ga111) allele is sensitive and reversible and we used this feature to test the effect of nicotine on the pumping rate. We would expect that ga111 worms grown at 20°C to be the most sensitive and most reversible, and for the sensitivity and reversibility to decrease as the temperature increases. Indeed, ga111 worms grown at 20°C were very sensitive to nicotine. Our data showed that both the high and even the low nicotine dose could significantly recover mpk-1 activation which in turn negatively affected pumping in this sensitive background (Figure 3.5A). When grown at 22°C and 25°C, the sensitivity and recovery decreased such that nicotine could no longer hyperactivate mpk-1 resulting in a close to normal pumping rate as observed at 22°C, or alleviated the inhibition by 25% when grown at 25°C relative to N2 (Figure 3.5A). The incomplete rescue at 25°C could be

due to the stress of the high temperature with the high nicotine dose, but it could still recue the inhibition in response to the less stressful low nicotine dose. For example, worms grown at 23-33°C accelerated paralysis in response to aldicarb (Kalinnikova T.B. et al., 2013). In addition, miR-80 was reported to be responsive to 25°C (Nehammer, Podolska, Mackowiak, Kagias, & Pocock, 2015) and that could have prevented the complete rescue observed when grown at 22°C. Lastly, the inhibition was also reduced by 42% when nicotine was co-treated with U0126 (Figure 3.5B). These data confirm previous reports that nicotine activates mpk-1 and that the hyperactivation of this pathway results in hypercontraction and consequently pumping inhibition.

You et al generated N2 worm lines with mpk-1 overexpressed in the pharynx and these lines had a severe feeding defect, slower growth rate, starvation phenotype and arrested at L1. They concluded that this hyperactive mpk-1 kinase phenocopied the grinder defect in cases of hyperactivated muscarinic signaling (You, Kim, Cobb, & Avery, 2006). This was not completely the case in our strains overexpressing mpk-1 in the pharynx. The difference could be attributed to the technical approach such that the lines generated by You et al were based on the injection of PCR fusion fragments (You et al., 2006). On the other hand, our construct was integrated into a plasmid and then injected to the worm's gonads. Transgenic lines were based on extrachromosomal mpk-1 and were characterized with low penetrance. Worms were transferred and grown and enriched over several generations before exposure to nicotine. GFP was fused downstream of mpk-1, therefore worms with pharyngeal mpk-1 overexpression were fluorescent and could be picked using a fluorescent stereoscope. Positive worms were shorter and grew slightly slower; however, they did not arrest at L1 and proceeded till adulthood to lay eggs and were superficially wild type. This was probably due to mpk-1 being expressed at a level that was just high enough, but below toxic levels which made it desirable for our experiments. We treated

those worms with pharyngeal mpk-1 overexpression with low and high doses of nicotine. This overexpression did not increase the severity of inhibition by the high nicotine dose, while it slightly increased the sensitivity of worms exposed to the low dose. Since it mirrored the original phenotype in N2 worms, it further confirmed that that the high nicotine did result in the hyperactivation of mpk-1 which locked the pharyngeal grinder and hindered normal pumping (You et al., 2006).

The relationship between miR-80, sir-2.1, and mpk-1 in nicotine-induced pumping response

We also overexpressed mpk-1 GFP fusion in the pharynx of miR-80 (*lof*) and sir-2.1 (*lof*) worms and checked the pumping rates in response to nicotine. We had established that mpk-1 hyperactivation results in pharyngeal pumping inhibition. If mpk-1 is downstream of miR-80 or sir-2.1, then overexpression of mpk-1 in the pharynx will reconstitute nicotine-induced pumping inhibition. Indeed, this is what we observed as pumping decreased in those backgrounds confirming that mpk-1 is downstream of either or both factors (Figure 3.5C).We then co-treated miR-80 (*lof*) and sir-2.1 (*lof*) worms with nicotine and the mpk-1 inhibitor U0126. We expected that this co-treatment would affect the pumping in neither of those backgrounds. Interestingly, the pumping slightly did decrease in response to the high nicotine dose in both backgrounds (Figure 3.5B). As a side note, U0126 nicotine co-treatment recovered nicotine-induced pharyngeal pumping inhibition by 42%. What about the remaining 25% inhibition? Either that U0126 concentration was insufficient to overcome the entire nicotine-induced mpk-1 hyperactivation or it could be that other factors might account for the remaining 25% inhibition.

We checked the function of U0126 and found that it is a specific non-competitive inhibitor for mek-2/MEK1/2 substrates, ATP and mpk-1/ERK1/2, and therefore inhibits mek-

2/MEK1/2's ability to phosphorylate (Favata et al., 1998). By affecting the ATP ratio, U0126 was also shown to activate aak-1/2/AMPK (Dokladda, Green, Pan, & Hardie, 2005). The authors suggested the need for caution when interpreting experiments conducted with U0126, and this helped us in understanding our results. aak-2/AMPK is inhibited by serotonin from the ADF neurons which results in glutamate release and consequent increase in pumping rate. In aak-1/2 loss of function worms, pumping increased and its over-activation decreased pumping (Cunningham et al., 2012). aak-2/AMPK.is also activated by nicotine (S. Wang et al., 2012). This means that nicotine and U0126 potentiated the activation of aak-2/AMPK and that would explain the incomplete recovery we observe in N2 background (25% inhibition) and miR-80 (*lof*) (26% inhibition) (Figure 3.5B). Meanwhile, there was a 43% inhibition when sir-2.1 (*lof*) worms were co-treated with nicotine and U0126 (Figure 3.5B). Why was this reduction more severe? It could be that (i) aak-2/AMPK is higher, or (ii) mpk-1 activation is lower in sir-2.1 (*lof*) background. aak-2/AMPK activation is less likely to be hyperactivated in sir-2.1 (*lof*) because aak-2 and sir-2.1 activate each other (Ruderman et al., 2010). The second possibility is more likely and suggests that sir-2.1 is an upstream regulator. This is supported by our data such that the percent inhibition in sir-2.1 (*lof*) (43%) was similar to the percent recovery of N2 (67%-25%=42%) when mpk-1 was inhibited my U0126 (Figure 3.5B). Therefore, nicotine might activate sir-2.1 which in turn activates mpk-1 directly and indirectly through miR-80. Whether or not nicotine-induced aak-2/AMPK activation is sufficient to contribute to 25% of pumping inhibition remains unconfirmed and this could be tested by treating temperature sensitive aak-2 (*lof*) mutants with nicotine without U0126. Interesting, serotonin production was reduced by 33% in response to the high nicotine dose (p=0.01) (Figure 3.6) and this supports the idea that the

high nicotine dose decreases serotonin signaling which increases aak-2/AMPK and further inhibits pumping.

In conclusion, this is the first report to show sir-2.1 and miR-80 as upstream positive regulators of mpk-1 hyperactivation in response to early nicotine exposure. While none of those effectors could rescue nicotine-induced developmental delay, our results unexpectedly brought to light aak-2/AMPK as a potential candidate which is activated when energy production is compromised or when energy consumption increases like in cases of increased muscle contraction (Jager, Handschin, St-Pierre, & Spiegelman, 2007; Wallace & Fan, 2010). The latter is considered a metabolic master switch that prevents ATP consumption and activates ATP production (Beale, 2008). aak-2/AMPK mutants were L1 arrest defective such that they initiated post-embryonic development even in the absence of food (Baugh & Sternberg, 2006). aak-2/AMPK was also required for inhibition of germline stem cell division during L1 arrest (Fukuyama et al., 2012). Nicotine activated aak-2/AMPK and consequently increased abdominal aortic aneurysms in mice (S. Wang et al., 2012). Therefore, aak-2/AMPK might be a potential contributor to nicotine-induced developmental arrest. In addition, the cytochrome P450 might be involved in mediating the developmental delay. For example, loss of function mutation of cyp-35A partially rescued the developmental delay in response to high caffeine doses (Min, Kawasaki, Gong, & Shim, 2015). Therefore cytochromes homologous to mammalian CYP2A6 (N. L. Benowitz et al., 2009) could contribute to nicotine-induced developmental delay.

References

Altun, Z. F., & Hall, D. H. (2009). Epithelial system, seam cells. . *WormAtlas*. doi: doi:10.3908/wormatlas.1.14

Alvarez-Saavedra, E., & Horvitz, H. R. (2010). Many families of *C. elegans* microRNAs are not essential for development or viability. [Research Support, Non-U.S. Gov't]. *Curr Biol, 20*(4), 367-373. doi: 10.1016/j.cub.2009.12.051

Avery, L., & Horvitz, H. R. (1990). Effects of starvation and neuroactive drugs on feeding in Caenorhabditis elegans. *J Exp Zool, 253*(3), 263-270. doi: 10.1002/jez.1402530305

Avery, L., & You, Y. J. (2012). *C. elegans* feeding. *WormBook*, 1-23. doi: 10.1895/wormbook.1.150.1

Bamps, S., Wirtz, J., Savory, F. R., Lake, D., & Hope, I. A. (2009). The Caenorhabditis elegans sirtuin gene, sir-2.1, is widely expressed and induced upon caloric restriction. [Research Support, Non-U.S. Gov't]. *Mech Ageing Dev, 130*(11-12), 762-770. doi: 10.1016/j.mad.2009.10.001

Bargmann, C. I. (2006). Chemosensation in *C. elegans*. [Review]. *WormBook*, 1-29. doi: 10.1895/wormbook.1.123.1

Bates, E. A., Victor, M., Jones, A. K., Shi, Y., & Hart, A. C. (2006). Differential contributions of Caenorhabditis elegans histone deacetylases to huntingtin polyglutamine toxicity. *J Neurosci, 26*(10), 2830-2838. doi: 10.1523/JNEUROSCI.3344-05.2006

Baugh, L. R. (2013). To grow or not to grow: nutritional control of development during Caenorhabditis elegans L1 arrest. *Genetics, 194*(3), 539-555. doi: 10.1534/genetics.113.150847

Baugh, L. R., & Sternberg, P. W. (2006). DAF-16/FOXO regulates transcription of cki-1/Cip/Kip and repression of lin-4 during *C. elegans* L1 arrest. *Curr Biol, 16*(8), 780-785. doi: 10.1016/j.cub.2006.03.021

Beale, E. G. (2008). 5'-AMP-activated protein kinase signaling in Caenorhabditis elegans. *Exp Biol Med (Maywood), 233*(1), 12-20. doi: 10.3181/0705-MR-117

Becatti, M., Fiorillo, C., Barygina, V., Cecchi, C., Lotti, T., Prignano, F., . . . Taddei, N. (2014). SIRT1 regulates MAPK pathways in vitiligo skin: insight into the molecular pathways of cell survival. [Research Support, Non-U.S. Gov't]. *J Cell Mol Med, 18*(3), 514-529. doi: 10.1111/jcmm.12206

Becatti, M., Taddei, N., Cecchi, C., Nassi, N., Nassi, P. A., & Fiorillo, C. (2012). SIRT1 modulates MAPK pathways in ischemic-reperfused cardiomyocytes. [Research Support, Non-U.S. Gov't]. *Cell Mol Life Sci, 69*(13), 2245-2260. doi: 10.1007/s00018-012-0925-5

Benowitz, N. L., Hukkanen, J., & Jacob, P., 3rd. (2009). Nicotine chemistry, metabolism, kinetics and biomarkers. *Handb Exp Pharmacol*(192), 29-60. doi: 10.1007/978-3-540-69248-5_2

Brennecke, J., Hipfner, D. R., Stark, A., Russell, R. B., & Cohen, S. M. (2003). bantam encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene hid in Drosophila. *Cell, 113*(1), 25-36.

Brunzell, D. H., Mineur, Y. S., Neve, R. L., & Picciotto, M. R. (2009). Nucleus accumbens CREB activity is necessary for nicotine conditioned place preference. *Neuropsychopharmacology, 34*(8), 1993-2001. doi: 10.1038/npp.2009.11

Cargnello, M., & Roux, P. P. (2011). Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. *Microbiol Mol Biol Rev, 75*(1), 50-83. doi: 10.1128/MMBR.00031-10

Chen, F., Mackerell, A. D., Jr., Luo, Y., & Shapiro, P. (2008). Using Caenorhabditis elegans as a model organism for evaluating extracellular signal-regulated kinase docking domain inhibitors. *J Cell Commun Signal, 2*(3-4), 81-92. doi: 10.1007/s12079-008-0034-2

Chikina, M. D., Huttenhower, C., Murphy, C. T., & Troyanskaya, O. G. (2009). Global prediction of tissue-specific gene expression and context-dependent gene networks in Caenorhabditis elegans. *PLoS Comput Biol, 5*(6), e1000417. doi: 10.1371/journal.pcbi.1000417

Conrad, M., Gorka, S. M., & Kassel, J. (2015). Smoking's effects on respiratory sinus arrhythmia in adolescent smokers. *Int J Psychophysiol, 97*(1), 8-13. doi: 10.1016/j.ijpsycho.2015.05.002

Cunningham, K. A., Hua, Z., Srinivasan, S., Liu, J., Lee, B. H., Edwards, R. H., & Ashrafi, K. (2012). AMP-activated kinase links serotonergic signaling to glutamate release for regulation of feeding behavior in *C. elegans*. *Cell Metab, 16*(1), 113-121. doi: 10.1016/j.cmet.2012.05.014

D'Alessandro, A., Boeckelmann, I., Hammwhoner, M., & Goette, A. (2012). Nicotine, cigarette smoking and cardiac arrhythmia: an overview. [Review]. *Eur J Prev Cardiol, 19*(3), 297-305.

de Lucas, M. P., Saez, A. G., & Lozano, E. (2015). miR-58 family and TGF-beta pathways regulate each other in Caenorhabditis elegans. [Research Support, Non-U.S. Gov't]. *Nucleic Acids Res, 43*(20), 9978-9993. doi: 10.1093/nar/gkv923

Dhillon, A. S., Hagan, S., Rath, O., & Kolch, W. (2007). MAP kinase signalling pathways in cancer. *Oncogene, 26*(22), 3279-3290. doi: 10.1038/sj.onc.1210421

Dokladda, K., Green, K. A., Pan, D. A., & Hardie, D. G. (2005). PD98059 and U0126 activate AMP-activated protein kinase by increasing the cellular AMP:ATP ratio and not via inhibition of the MAP kinase pathway. [Research Support, Non-U.S. Gov't]. *FEBS Lett, 579*(1), 236-240. doi: 10.1016/j.febslet.2004.11.084

Favata, M. F., Horiuchi, K. Y., Manos, E. J., Daulerio, A. J., Stradley, D. A., Feeser, W. S., . . . Trzaskos, J. M. (1998). Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J Biol Chem, 273*(29), 18623-18632.

Ferguson, D., Koo, J. W., Feng, J., Heller, E., Rabkin, J., Heshmati, M., . . . Nestler, E. J. (2013). Essential role of SIRT1 signaling in the nucleus accumbens in cocaine and morphine action. *J Neurosci, 33*(41), 16088-16098. doi: 10.1523/JNEUROSCI.1284-13.2013

Ferguson, D., Shao, N., Heller, E., Feng, J., Neve, R., Kim, H. D., . . . Nestler, E. J. (2015). SIRT1-FOXO3a regulate cocaine actions in the nucleus accumbens. *J Neurosci, 35*(7), 3100-3111. doi: 10.1523/JNEUROSCI.4012-14.2015

Fukuyama, M., Sakuma, K., Park, R., Kasuga, H., Nagaya, R., Atsumi, Y., . . . Katada, T. (2012). *C. elegans* AMPKs promote survival and arrest germline development during nutrient stress. *Biol Open, 1*(10), 929-936. doi: 10.1242/bio.2012836

Fusco, S., Ripoli, C., Podda, M. V., Ranieri, S. C., Leone, L., Toietta, G., . . . Pani, G. (2012). A role for neuronal cAMP responsive-element binding (CREB)-1 in brain responses to calorie restriction. [Research Support, Non-U.S. Gov't]. *Proc Natl Acad Sci U S A, 109*(2), 621-626. doi: 10.1073/pnas.1109237109

Gao, J., Wang, W. Y., Mao, Y. W., Graff, J., Guan, J. S., Pan, L., . . . Tsai, L. H. (2010). A novel pathway regulates memory and plasticity via SIRT1 and miR-134. *Nature, 466*(7310), 1105-1109. doi: 10.1038/nature09271

Goodman, M. B. (2006). Mechanosensation. *WormBook*, 1-14. doi: 10.1895/wormbook.1.62.1
Greiss, S., Hall, J., Ahmed, S., & Gartner, A. (2008). *C. elegans* SIR-2.1 translocation is linked to a proapoptotic pathway parallel to cep-1/p53 during DNA damage-induced apoptosis. [Research Support, Non-U.S. Gov't]. *Genes Dev, 22*(20), 2831-2842. doi: 10.1101/gad.482608
Halevi, S., McKay, J., Palfreyman, M., Yassin, L., Eshel, M., Jorgensen, E., & Treinin, M. (2002). The *C. elegans* ric-3 gene is required for maturation of nicotinic acetylcholine receptors. *EMBO J, 21*(5), 1012-1020. doi: 10.1093/emboj/21.5.1012
Jager, S., Handschin, C., St-Pierre, J., & Spiegelman, B. M. (2007). AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1alpha. *Proc Natl Acad Sci U S A, 104*(29), 12017-12022. doi: 10.1073/pnas.0705070104
Kalinnikova T.B., Shagidullin R.R., Kolsanova R.R., Osipova E.B., Zakharov S.V., & M.K., G. (2013). Acetylcholine Deficiency in Caenorhabditis elegans Induced by Hyperthermia Can Be Compensated by ACh-esterase Inhibition or Activation of GAR-3 mAChRs. *Environment and Natural Resources Research, 3*(3). doi: http://dx.doi.org/10.5539/enrr.v3n3p98
Kawakita A, Sato K, Makino H, Ikegami H, & Takayama S, e. a. (2008). Nicotine Acts on Growth Plate Chondrocytes to Delay Skeletal Growth through the α7 Neuronal Nicotinic Acetylcholine Receptor. *PLoS ONE, 3*(12), e3945. doi: doi:10.1371/journal.pone.0003945
Kim, E. K., & Choi, E. J. (2010). Pathological roles of MAPK signaling pathways in human diseases. *Biochim Biophys Acta, 1802*(4), 396-405. doi: 10.1016/j.bbadis.2009.12.009
Kimura, Y., Corcoran, E. E., Eto, K., Gengyo-Ando, K., Muramatsu, M. A., Kobayashi, R., . . . Tokumitsu, H. (2002). A CaMK cascade activates CRE-mediated transcription in neurons of Caenorhabditis elegans. *EMBO Rep, 3*(10), 962-966. doi: 10.1093/embo-reports/kvf191
Kwok, R. P., Lundblad, J. R., Chrivia, J. C., Richards, J. P., Bachinger, H. P., Brennan, R. G., . . . Goodman, R. H. (1994). Nuclear protein CBP is a coactivator for the transcription factor CREB. *Nature, 370*(6486), 223-226. doi: 10.1038/370223a0
Lackner, M. R., & Kim, S. K. (1998). Genetic analysis of the Caenorhabditis elegans MAP kinase gene mpk-1. [Research Support, U.S. Gov't, P.H.S.]. *Genetics, 150*(1), 103-117.
Lakhina, V., Arey, R. N., Kaletsky, R., Kauffman, A., Stein, G., Keyes, W., . . . Murphy, C. T. (2015). Genome-wide functional analysis of CREB/long-term memory-dependent transcription reveals distinct basal and memory gene expression programs. *Neuron, 85*(2), 330-345. doi: 10.1016/j.neuron.2014.12.029
Li, C. Y., Mao, X., & Wei, L. (2008). Genes and (common) pathways underlying drug addiction. *PLoS Comput Biol, 4*(1), e2. doi: 10.1371/journal.pcbi.0040002
Li, Y., Xu, W., McBurney, M. W., & Longo, V. D. (2008). SirT1 inhibition reduces IGF-I/IRS-2/Ras/ERK1/2 signaling and protects neurons. *Cell Metab, 8*(1), 38-48. doi: 10.1016/j.cmet.2008.05.004
Matta, S. G., Balfour, D. J., Benowitz, N. L., Boyd, R. T., Buccafusco, J. J., Caggiula, A. R., . . . Zirger, J. M. (2007). Guidelines on nicotine dose selection for in vivo research. *Psychopharmacology (Berl), 190*(3), 269-319. doi: 10.1007/s00213-006-0441-0
Mayr, B., & Montminy, M. (2001). Transcriptional regulation by the phosphorylation-dependent factor CREB. [Review]. *Nat Rev Mol Cell Biol, 2*(8), 599-609. doi: 10.1038/35085068

McKay, J. P., Raizen, D. M., Gottschalk, A., Schafer, W. R., & Avery, L. (2004). eat-2 and eat-18 are required for nicotinic neurotransmission in the Caenorhabditis elegans pharynx. [Research Support, U.S. Gov't, P.H.S.]. *Genetics, 166*(1), 161-169.

Min, H., Kawasaki, I., Gong, J., & Shim, Y. H. (2015). Caffeine induces high expression of cyp-35A family genes and inhibits the early larval development in Caenorhabditis elegans. [Research Support, Non-U.S. Gov't]. *Mol Cells, 38*(3), 236-242. doi: 10.14348/molcells.2015.2282

Monsalve, G. C., Van Buskirk, C., & Frand, A. R. (2011). LIN-42/PERIOD controls cyclical and developmental progression of *C. elegans* molts. *Curr Biol, 21*(24), 2033-2045. doi: 10.1016/j.cub.2011.10.054

Nehammer, C., Podolska, A., Mackowiak, S. D., Kagias, K., & Pocock, R. (2015). Specific microRNAs regulate heat stress responses in Caenorhabditis elegans. *Sci Rep, 5*, 8866. doi: 10.1038/srep08866

Noriega, L. G., Feige, J. N., Canto, C., Yamamoto, H., Yu, J., Herman, M. A., . . . Auwerx, J. (2011). CREB and ChREBP oppositely regulate SIRT1 expression in response to energy availability. *EMBO Rep, 12*(10), 1069-1076. doi: 10.1038/embor.2011.151

Okuyama, T., Inoue, H., Ookuma, S., Satoh, T., Kano, K., Honjoh, S., . . . Nishida, E. (2010). The ERK-MAPK pathway regulates longevity through SKN-1 and insulin-like signaling in Caenorhabditis elegans. [Research Support, Non-U.S. Gov't]. *J Biol Chem, 285*(39), 30274-30281. doi: 10.1074/jbc.M110.146274

Olson, E. N. (2006). Gene regulatory networks in the evolution and development of the heart. *Science, 313*(5795), 1922-1927. doi: 10.1126/science.1132292

Rahman, S., & Islam, R. (2011). Mammalian Sirt1: insights on its biological functions. *Cell Commun Signal, 9*, 11. doi: 10.1186/1478-811X-9-11

Renthal, W., Kumar, A., Xiao, G., Wilkinson, M., Covington, H. E., 3rd, Maze, I., . . . Nestler, E. J. (2009). Genome-wide analysis of chromatin regulation by cocaine reveals a role for sirtuins. [Research Support, N.I.H., Extramural]. *Neuron, 62*(3), 335-348. doi: 10.1016/j.neuron.2009.03.026

Robison, A. J., & Nestler, E. J. (2011). Transcriptional and epigenetic mechanisms of addiction. *Nat Rev Neurosci, 12*(11), 623-637. doi: 10.1038/nrn3111

Ruderman, N. B., Xu, X. J., Nelson, L., Cacicedo, J. M., Saha, A. K., Lan, F., & Ido, Y. (2010). AMPK and SIRT1: a long-standing partnership? *Am J Physiol Endocrinol Metab, 298*(4), E751-760. doi: 10.1152/ajpendo.00745.2009

Rutkowski, R., Dickinson, R., Stewart, G., Craig, A., Schimpl, M., Keyse, S. M., & Gartner, A. (2011). Regulation of Caenorhabditis elegans p53/CEP-1-dependent germ cell apoptosis by Ras/MAPK signaling. [Research Support, Non-U.S. Gov't]. *PLoS Genet, 7*(8), e1002238. doi: 10.1371/journal.pgen.1002238

Saur, T., DeMarco, S. E., Ortiz, A., Sliwoski, G. R., Hao, L., Wang, X., . . . Buttner, E. A. (2013). A genome-wide RNAi screen in Caenorhabditis elegans identifies the nicotinic acetylcholine receptor subunit ACR-7 as an antipsychotic drug target. *PLoS Genet, 9*(2), e1003313. doi: 10.1371/journal.pgen.1003313

Stiernagle, T. (2006). Maintenance of *C. elegans*. . *WormBook: The Online Review of C. elegans Biology [Internet]*. *Pasadena (CA): WormBook; 2005-. Available from: http://www.ncbi.nlm.nih.gov/books/NBK19649/*.

Sugi, T., Ohtani, Y., Kumiya, Y., Igarashi, R., & Shirakawa, M. (2014). High-throughput optical quantification of mechanosensory habituation reveals neurons encoding memory in

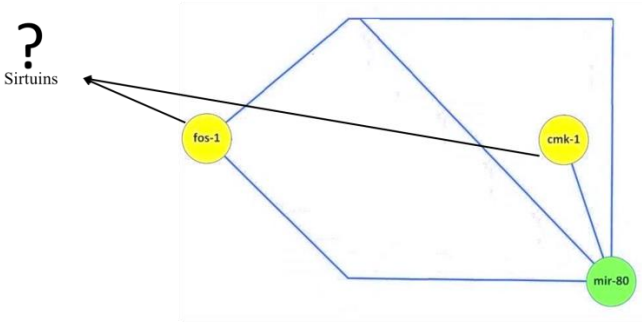
Caenorhabditis elegans. [Research Support, Non-U.S. Gov't]. *Proc Natl Acad Sci U S A, 111*(48), 17236-17241. doi: 10.1073/pnas.1414867111
Sundaram, M. V. (2006). RTK/Ras/MAPK signaling. *WormBook*, 1-19. doi: 10.1895/wormbook.1.80.1
Svoboda, K. R., Vijayaraghavan, S., & Tanguay, R. L. (2002). Nicotinic receptors mediate changes in spinal motoneuron development and axonal pathfinding in embryonic zebrafish exposed to nicotine. *J Neurosci, 22*(24), 10731-10741.
Taki, F., Pan, X., & Zhang, B. (2013). Nicotine Exposure Caused Significant Transgenerational Heritable Behavioral Changes in Caenorhabditis elegans. *Excli J., 12*(1611-2156), 793-806. doi: http://www.excli.de/vol12/Zhang_10092013_proof.pdf
Taki, F. A., Pan, X., Lee, M.-H., & Zhang, B. (2014). Nicotine exposure and transgenerational impact: a prospective study on small regulatory microRNAs. *Sci. Rep., 4*(7513). doi: DOI:10.1038/srep07513
Taki, F. A., Pan, X., & Zhang, B. (2013). Chronic nicotine exposure systemically alters microRNA expression profiles during post-embryonic stages in Caenorhabditis elegans. *J Cell Physiol, 229*(1), 79-89. doi: 10.1002/jcp.24419
Taki, F. A., & Zhang, B. (2013). Determination of reliable reference genes for multi-generational gene expression analysis on *C. elegans* exposed to abused drug nicotine. *Psychopharmacology (Berl)*, 1-12. doi: 10.1007/s00213-013-3139-0
Vassilopoulos, A., Fritz, K. S., Petersen, D. R., & Gius, D. (2011). The human sirtuin family: evolutionary divergences and functions. *Hum Genomics, 5*(5), 485-496.
Vora, M., Shah, M., Ostafi, S., Onken, B., Xue, J., Ni, J. Z., . . . Driscoll, M. (2013). Deletion of microRNA-80 activates dietary restriction to extend *C. elegans* healthspan and lifespan. *PLoS Genet, 9*(8), e1003737. doi: 10.1371/journal.pgen.1003737
Wallace, D. C., & Fan, W. (2010). Energetics, epigenetics, mitochondrial genetics. *Mitochondrion, 10*(1), 12-31. doi: 10.1016/j.mito.2009.09.006
Wang, S., Zhang, C., Zhang, M., Liang, B., Zhu, H., Lee, J., . . . Zou, M. H. (2012). Activation of AMP-activated protein kinase alpha2 by nicotine instigates formation of abdominal aortic aneurysms in mice in vivo. *Nat Med, 18*(6), 902-910. doi: 10.1038/nm.2711
Wright, D. A., Thibodeau-Beganny, S., Sander, J. D., Winfrey, R. J., Hirsh, A. S., Eichtinger, M., . . . Joung, J. K. (2006). Standardized reagents and protocols for engineering zinc finger nucleases by modular assembly. *Nat Protoc, 1*(3), 1637-1652. doi: 10.1038/nprot.2006.259
Xie, Y. B., Park, J. H., Kim, D. K., Hwang, J. H., Oh, S., Park, S. B., . . . Choi, H. S. (2009). Transcriptional corepressor SMILE recruits SIRT1 to inhibit nuclear receptor estrogen receptor-related receptor gamma transactivation. [Research Support, Non-U.S. Gov't]. *J Biol Chem, 284*(42), 28762-28774. doi: 10.1074/jbc.M109.034165
You, Y. J., Kim, J., Cobb, M., & Avery, L. (2006). Starvation activates MAP kinase through the muscarinic acetylcholine pathway in Caenorhabditis elegans pharynx. *Cell Metab, 3*(4), 237-245. doi: 10.1016/j.cmet.2006.02.012
Zhang, X., Zabinsky, R., Teng, Y., Cui, M., & Han, M. (2011). microRNAs play critical roles in the survival and recovery of Caenorhabditis elegans from starvation-induced L1 diapause. *Proc Natl Acad Sci U S A, 108*(44), 17997-18002. doi: 10.1073/pnas.1105982108
Zhao, Y., Luo, P., Guo, Q., Li, S., Zhang, L., Zhao, M., . . . Fei, Z. (2012). Interactions between SIRT1 and MAPK/ERK regulate neuronal apoptosis induced by traumatic brain injury in

vitro and in vivo. [Research Support, Non-U.S. Gov't]. *Exp Neurol*, *237*(2), 489-498. doi: 10.1016/j.expneurol.2012.07.004

Table 3.1: The effect of 30 hour post-embryonic nicotine exposure on seam cells per worm side in JR667 strain.

	N	Mean seam cells/side	Std. Error	Median seam cells/side	SD	Min	Max
Control	43	25.44	0.87	28.00	5.70	16	35
Low	39	23.21	0.90	25.00	5.63	16	32
High	55	12.33	0.56	11.00	4.15	9	28

(A)



KARG-based common molecular pathways	Nicotine
Neuroactive ligand-receptor interaction	7.79E-03**
Long-term potentiation	8.84E-03**
GnRH signaling pathway	1.72E-02*
MAPK signaling pathway	1.10E-02*
Gap junctions	5.85E-03**

miR-80 KEGG enrichment

Category	Term	P-Value
KEGG_PATHWAY: cel04330	Notch signaling pathway	0.0255
KEGG_PATHWAY: cel04010	MAPK signaling pathway	0.0494
KEGG_PATHWAY: cel04012	ErbB signaling pathway	0.0819

(B)

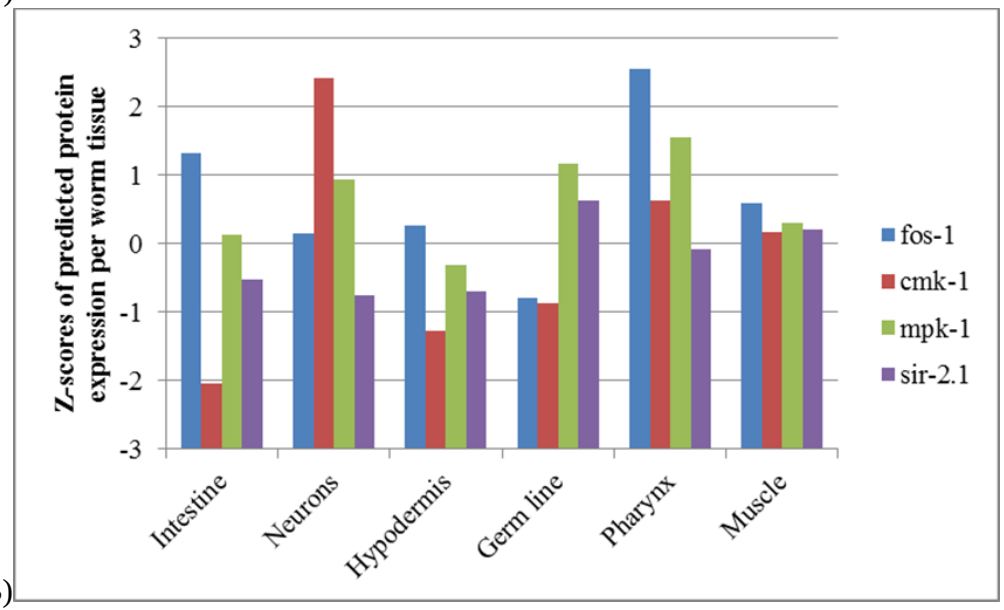
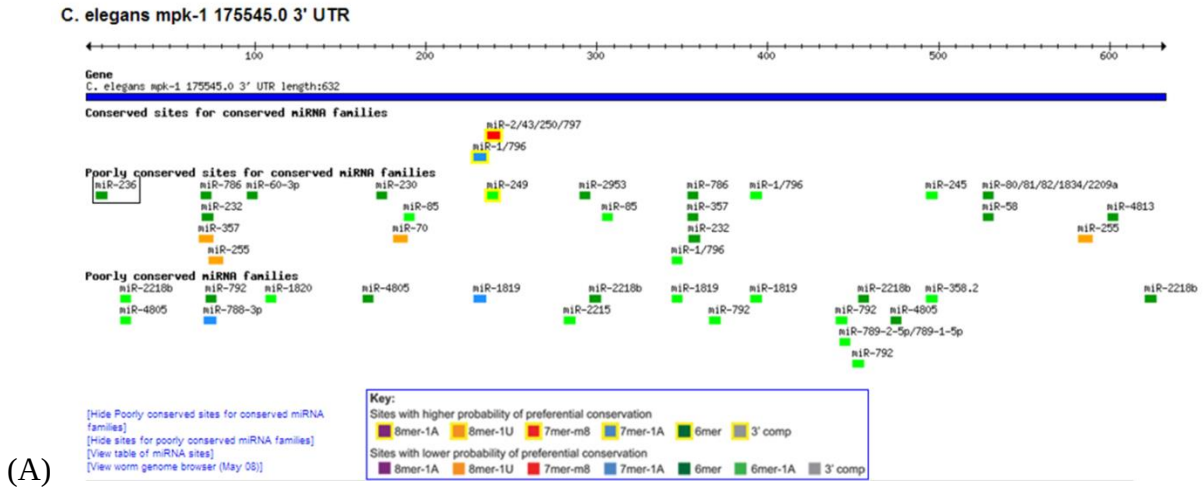
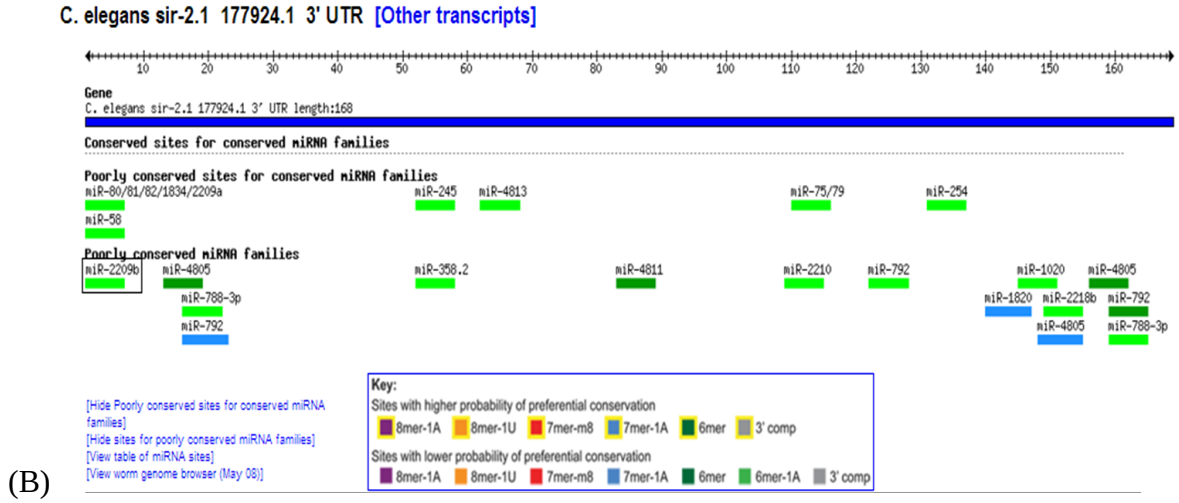


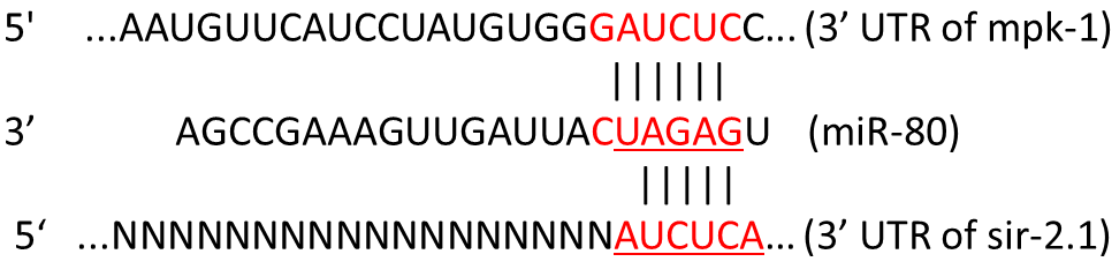
Figure 3.1: (A) Rational for MS80 (mpk-1, sir-2.1, miR-80). (B) Predictions for protein expression in different *C. elegans* tissue based on the program developed by Chikina et al. (Chikina et al., 2009).



(A)



(B)



(C)

Figure 3.2: TargetScanWorm (6.2)-based predictions for miRNA targets sites in (A) mpk-1, and (B) sir-2.1. (C) Predicted miR-80 MREs (miRNA response elements) in mpk-1and sir-2.1(Jan, Friedman, Ruby, & Bartel, 2011; B. P. Lewis, Burge, & Bartel, 2005).

(A)

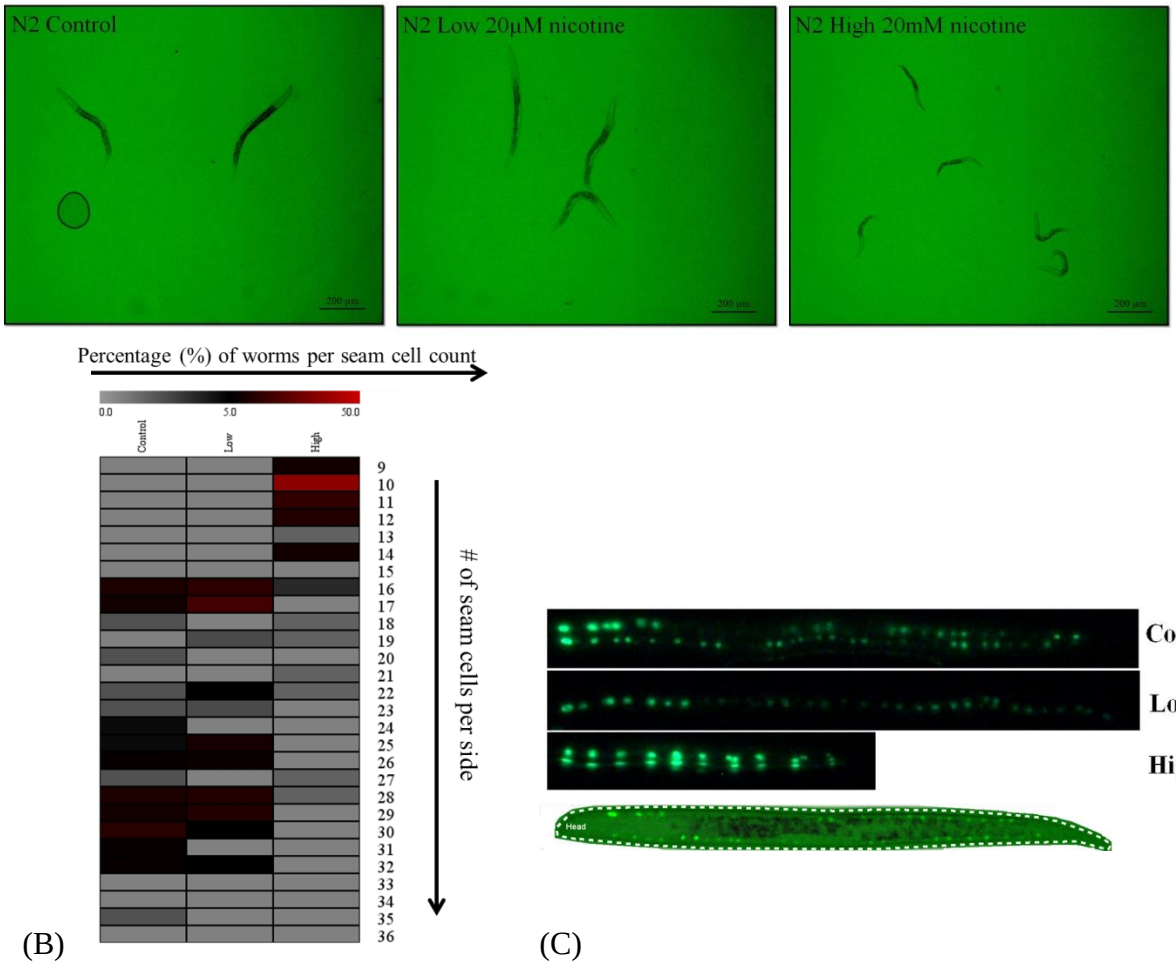


Figure 3.3: (A) Postembryonic nicotine exposure inhibited development in *C. elegans* N2. (B; C) Nicotine affected the normal timing of seam cell division in *C. elegans* (JR667 strain). (B) Heatmap: From left to right: Control (1st column), Low (2nd column), High (3rd column).

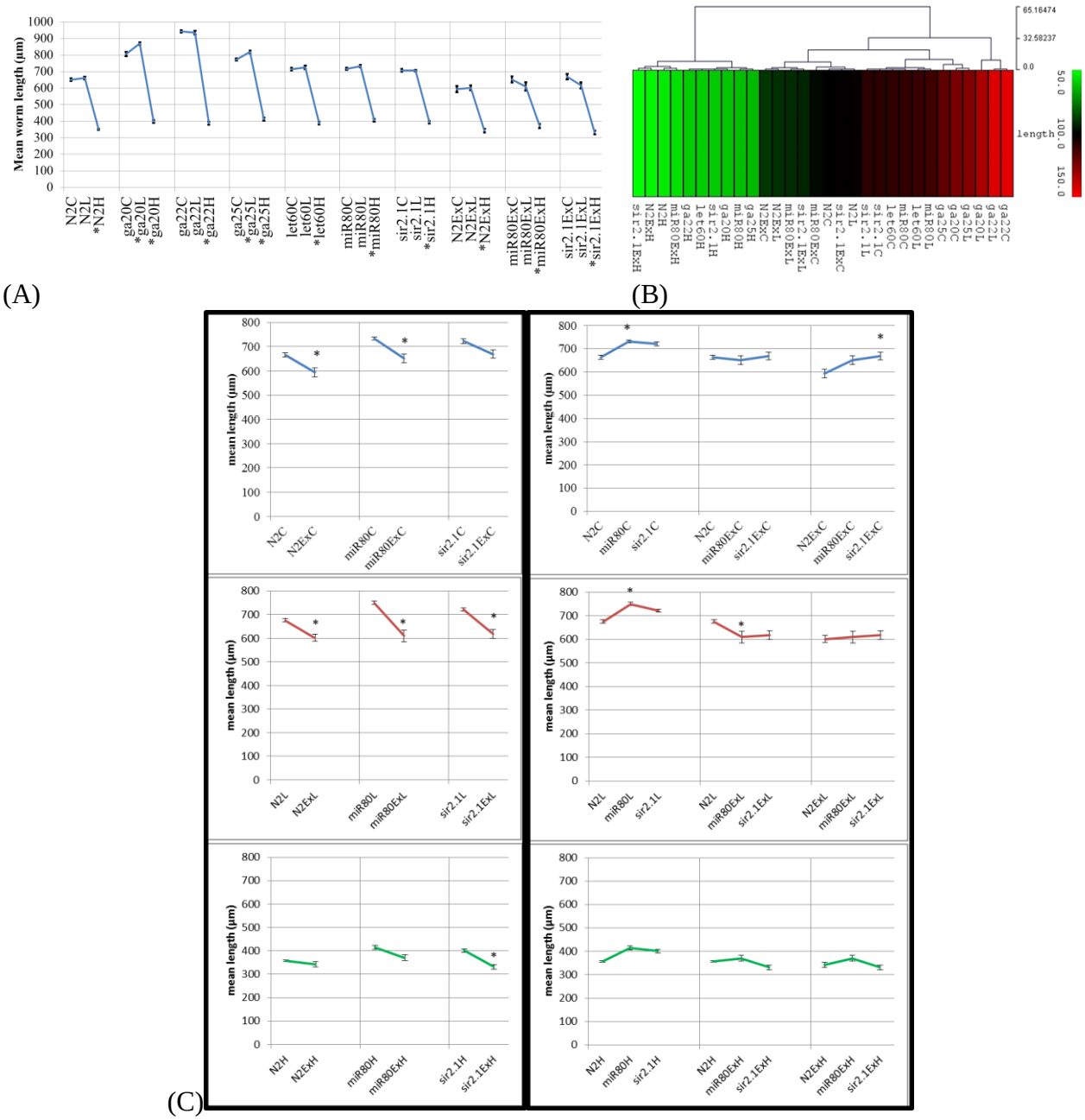


Figure 3.4: (A) The effect of post-embryonic nicotine exposure on worm length in different genetic backgrounds. (*) denotes statistically significant differences at $p<0.05$ relative to control per strain. (B) Heat map of the worm lengths in different genetic backgrounds normalized to the control group of the N2 lab strain and presented as percentages. Unsupervised hierarchical clustering was based on Euclidean distance and average linkage as described in the methods section. (C) Left column: The effect of pharyngeal overexpression of mpk-1 on worm length in each treatment group: control, low, high. Right column: comparisons of the nicotine-induced length patterns in N2, miR-80 (nDf53), sir-2.1 (ok434) with or without pharyngeal mpk-1 overexpression. (*) denotes statistically significant differences at $p<0.05$.

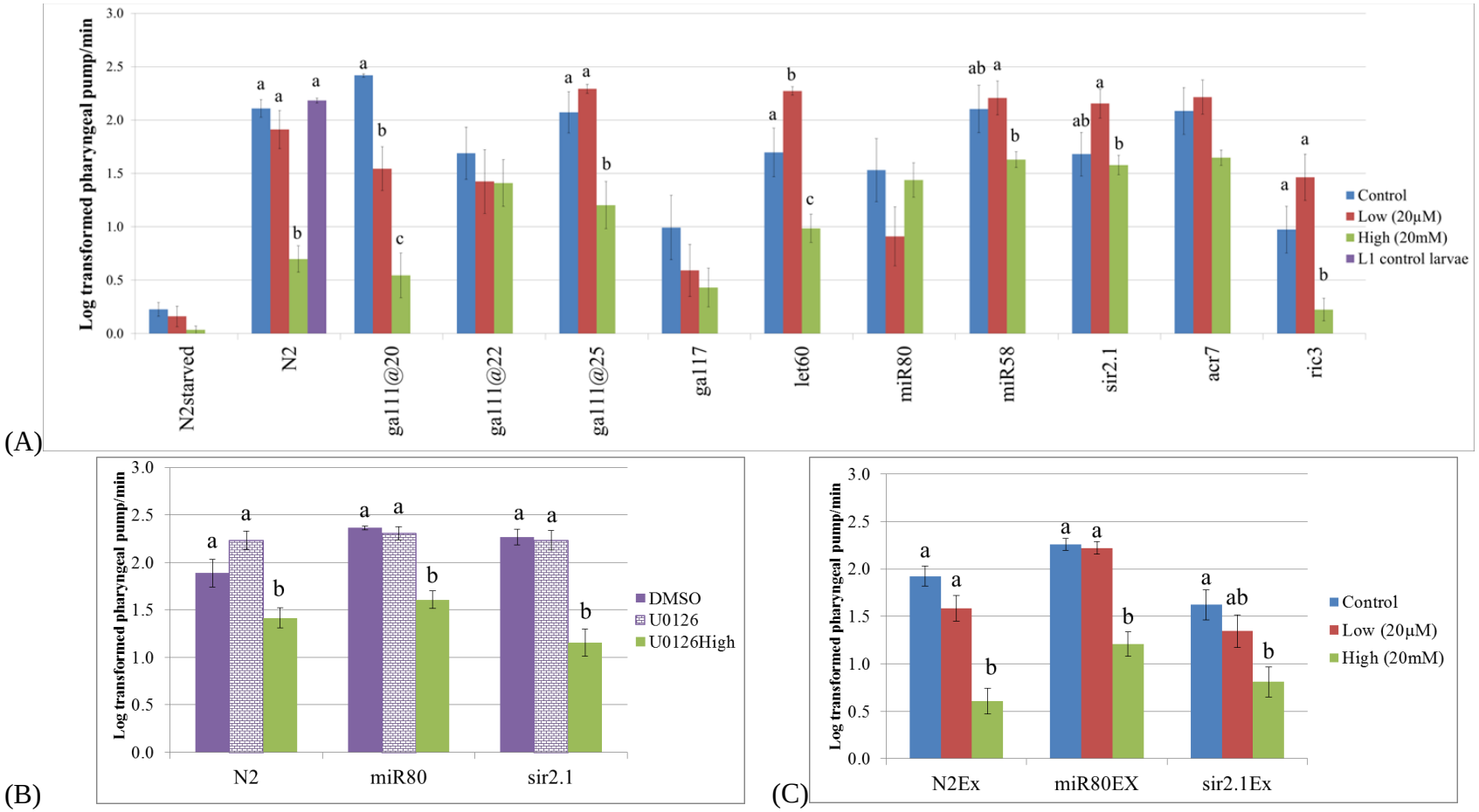
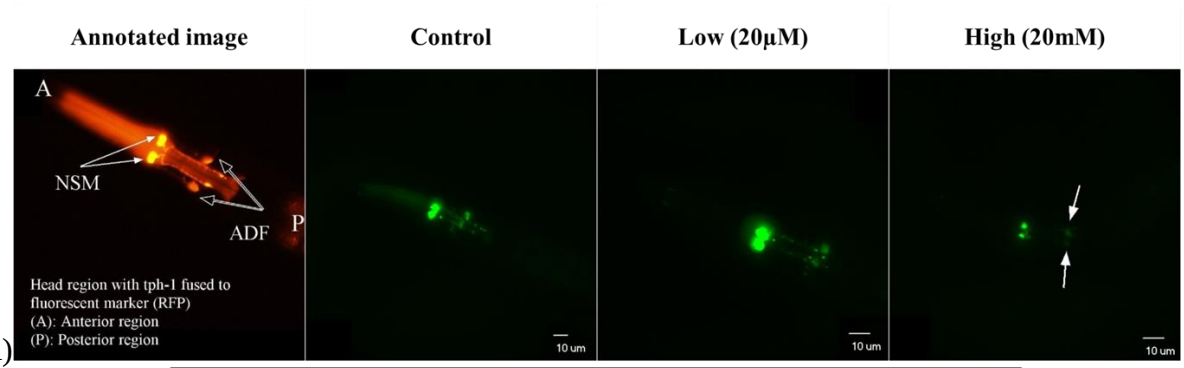
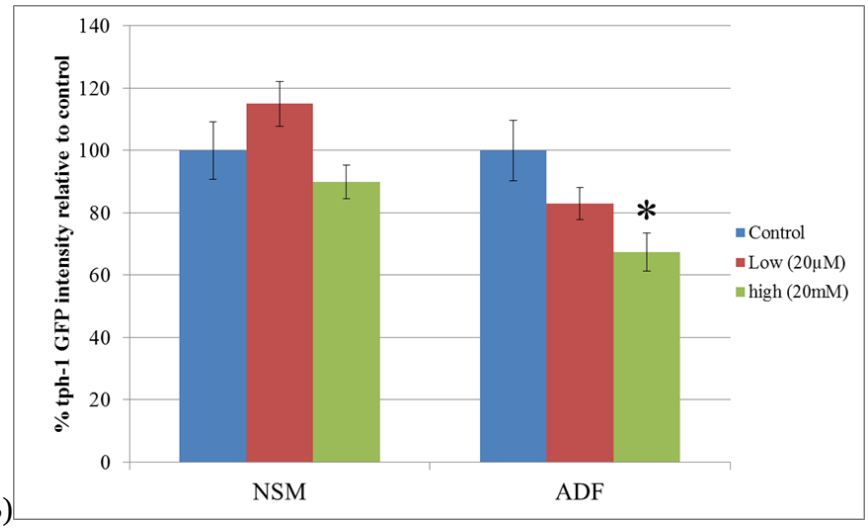


Figure 3.5: (A) The effect of postembryonic nicotine exposure on pharyngeal pumping in different genetic backgrounds. (B) The effect of U0126 (mpk-1 inhibitor) co-treatment on pharyngeal pumping in N2, miR-80 (NDf53), and sir-2.1 (ok434). (C) The effect of postembryonic nicotine exposure on N2, miR-80 (nDf53), and sir-2.1 (ok434) with pharyngeal-specific overexpression of mpk-1. Comparisons were done to the control of each strain. Different letters denote statistically significant differences at $p<0.05$.



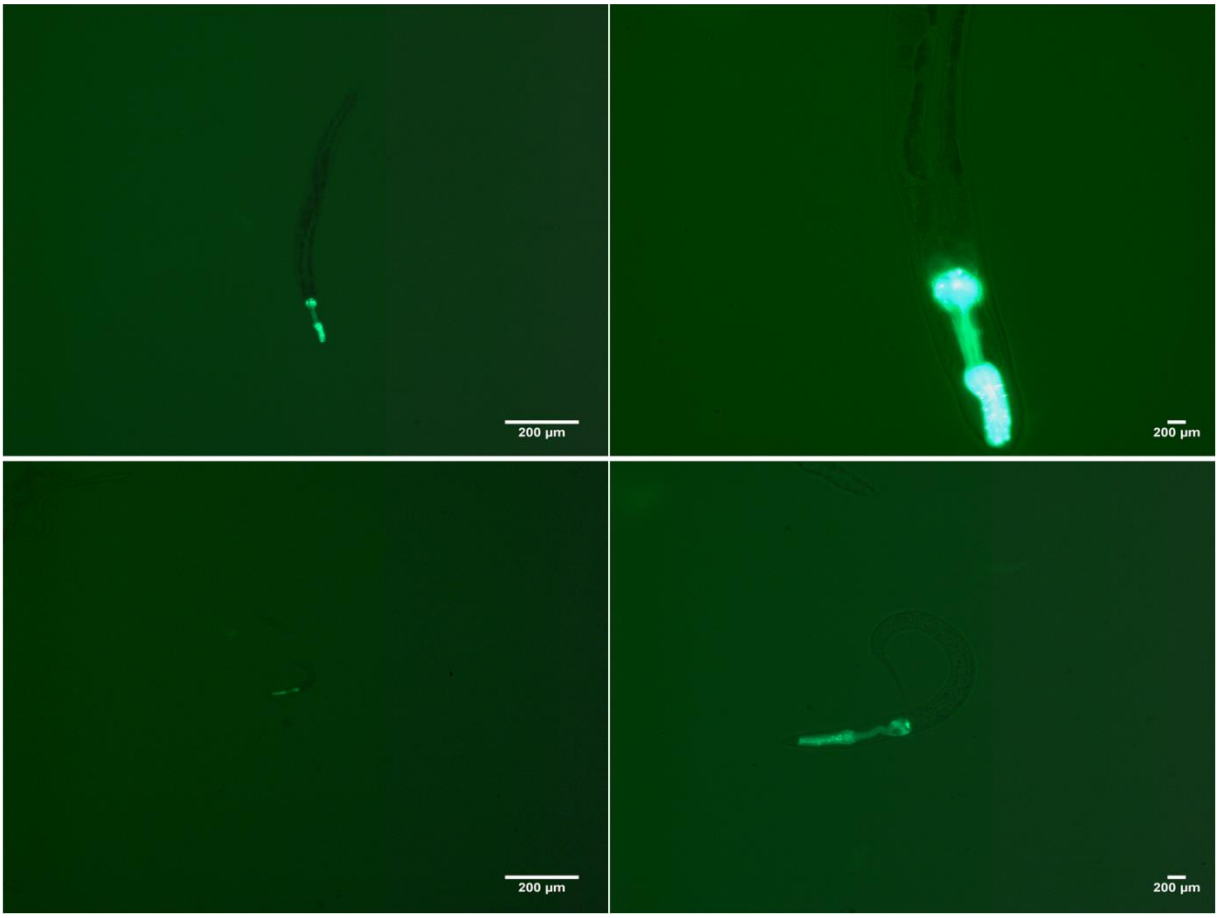
(A)



(B)

Figure 3.6: Postembryonic to the high nicotine exposure decreased serotonin synthesis in ADF neurons, but not NSM neurons at $p<0.05$. GR1366 worms have tph-1 tagged with GFP. tph-1 is the rate limiting enzyme for serotonin synthesis. Expression was based on GFP expression and was measured by imageJ software and was normalized to the area.

Supplementary



Supplementary figure 3.1: pharyngeal specific mpk-1 overexpression in adult worms (first row taken at 10X and 40X, respectively) and in L1 worms (second row taken at 10X and 40X, respectively).

Chapter IV

MS80: Fasting-induced resistance against late-onset nicotine-induced germ line apoptosis

Abstract

Nicotine remains the most socially accepted drug of abuse worldwide. This results in children and adolescents being exposed to nicotine either through active smoking or through second and even third hand smoking sources. This early exposure does not only impact the young individuals, but can also impact their future health. Unfortunately, the developmental molecular origins of the related late-onset diseases remain elusive. Therefore, in this study we were interested in investigating the contributions of mpk-1, sir-2.1, and miR-80 (MS80) to nicotine-induced enduring impacts using the model organism *C. elegans*. Worms were exposed to nicotine prior to sexual maturity and were then washed and transferred to grow on nicotine-free NGM plates until adulthood to examine their reproductive success, germ line apoptosis rate, and their average lifespan. One-way ANOVA and post-hoc test were used to test the impact of nicotine on reproduction and germ line apoptosis while Kaplan-Meier curves and Chi-square tests were used for lifespan analysis. Statistical significance was based on $p<0.05$ after applying Bonferroni corrections. Our results showed that early nicotine exposure delayed the onset of reproduction, but it did not decrease the fertility. In addition, this early exposure decreased mean worm lifespan by 5% and this effect was lost in miR-80 (*lof*) worms. Post-embryonic treatment with either the low (20μM) or the high (20mM) nicotine doses also increased adult germ line apoptosis rate and this was completely recovered in miR-80 (*lof*) worms and partially recovered in sir-2.1 (*lof*) worms. The apoptosis rate also went back to normal after treating worms with mpk-1 inhibitor (U0126). Interestingly, starvation concurrent with nicotine treatment made the worms resistant to nicotine-induced adult germ line apoptosis. Similarly, modeling a DR-like state after mpk-1 overexpression in the pharynx also rescued the apoptosis phenotype after

nicotine exposure. This was supported by epidemiological observations where annual fasting habits could explain the discrepancies between lower death rates in spite of the higher smoking prevalence. Our study is the first to show that MS80 mediate nicotine-induced late-onset germ line apoptosis and that manipulating this pathway can enhance an organism-level response that may boost the antioxidant system in a DR-like similar mechanism. This can prevent the susceptibilities of late-onset health disparities like neurodegenerative diseases related to early nicotine exposure.

Keywords: Nicotine, MAPK, ERK1/2, mpk-1, sirtuins, SIRT1, sir-2.1, miR-80, C. elegans larvae, neonates, adolescents, late-onset, lifespan, germ line apoptosis, ROS, oxidative damage, antioxidant system, prevention

Introduction

Though it is variable in different cultures, nicotine remains the most socially accepted drug of abuse worldwide (Goriounova & Mansvelder, 2012). For example, smoking rates among 11 year olds in Lebanon is above 60% (WHO, 2013). Smoking rates among adolescents are also increasing in the US due to the spread of waterpipes (i.e. hookas) and other nicotine products (Soule et al., 2015). Also, about 16% of women smoke during and after pregnancy or use nicotine replacement therapy in the US (Smith et al., 2010) and this subjects their developing embryo as well as the lactating neonates to nicotine through breast milk. Unfortunately, smokers remain unaware of the severity of their habit on their health as well as their family or continue to relapse due to the addictive nature of nicotine (Zhou et al., 2014). This results in children and adolescents being exposed to nicotine either through active smoking or through second and even third hand smoking sources (Martins-Green et al., 2014). Second hand smoke was linked to increased risks of neurodevelopmental delays and poor neurocognitive performances when experienced during the postnatal period (R. Chen et al., 2013). On a cellular level, nicotine exposure negatively impacted the survival of hippocampal progenitor cells in developing as well as adult brains (Berger, Gage, & Vijayaraghavan, 1998) and increased the apoptotic markers in the developing piglet brains (Machaalani, Waters, & Tinworth, 2005). This early exposure does not only impact the young individuals, but can also impose catastrophic impacts on their future health (Cuthbertson & Britton, 2010). Epidemiologic studies linked smoking with increased risks of neurodegenerative diseases like Parkinson's (Hernan et al., 2002) and Alzheimer's (Durazzo et al., 2014). In addition, the prevalence of Attention Deficit Hyperactivity Disorder (ADHD) autism cases has increased in the last years. Both neurologic diseases have an apoptotic component (Fredriksson & Archer, 2004; Wei et al., 2014) and some reports associated their

onset with exposure to parental smoking (James, 2013; Smith et al., 2010; Zhou et al., 2014). Recently, there has been an increased interest in and emphasis on understanding the mechanisms contributing to late-onset diseases in response to chemical and environmental exposures during sensitive developmental windows (Haugen et al., 2015; Wadhwa, Buss, Entringer, & Swanson, 2009). Interestingly, both DOHaD and epigenetics are prioritized areas of research in the NIHES strategic plan and include DNA and histone methylation as well as regulatory RNA molecules like microRNAs (Haugen et al., 2015). In this study, we were interested in investigating the role of microRNAs in mediating late-onset diseases in response to post-embryonic exposure in *C. elegans*.

Previously, we showed that early nicotine exposure was associated with changes in behavior and microRNA profiles across generations (F. Taki et al., 2013; F. A. Taki et al., 2014; F. A. Taki et al., 2013). We identified miR-80 as a potential transgenerational biomarker for early parental nicotine exposure. In chapter III, we showed that miR-80 alleviated nicotine-induced pharyngeal tetanus. Our results suggested a potential relationship between miR-80, mpk-1, and sir-2.1 in response to direct nicotine exposure. We were intrigued to test if this relationship mediates nicotine-induced late-onset diseases in *C. elegans*. In this study, we study the role of miR-80, mpk-1, and sir-2.1 in late-onset diseases in response to early nicotine exposure.

Material and methods

Strains from CGC: N2 (lab WT strain), MT2124 (let-60 n1046), MT13949 (mir-80(nDf53)III), MT15563 (nDf53 III; mir-58(n4640) IV; nDf54 X), VC199 (sir-2.1 (ok434) IV); FX863 (acr-7(tm863) II), RM509 (ric-3(md158) IV), MT1522 (ced-3(n717) IV), and TJ1 (cep-1(gk138) I),

MD701(bcIs39 [lim-7p::ced-1::GFP + lin-15(+)]. In addition, N2Ex, miR-80(nDf53)Ex, and sir-2.1(ok434)Ex were generated as previously described.

Worm maintenance and treatment

Worms were maintained on solid NGM (0.3% NaCl, 0.25% peptone, 1.7% agar, 0.5% cholesterol, 0.1% of 1M MgSO4, 0.1% of 1M CaCl2, and 2.5% of 1M KPO4) at 20°C. For treatment, we used two nicotine doses based on our previous studies (F. Taki et al., 2013; F. A. Taki et al., 2014; F. A. Taki et al., 2013; F. A. Taki & Zhang, 2013). Nicotine was diluted in the same KPO4 buffer used in NGM. A serial dilution was done to two nicotine stocks of 1M and 1mM. The latter were added during the making of the treatment plates such that the final nicotine concentrations in the media were 20mM (high dose) and 20µM (low dose) (F. A. Taki et al., 2014). Plates were left to dry, sealed, and stored at 4°C until use.

Worms used for experiments were first mixed with a freshly prepared synchronization solution ((70% water, 10% NaOH, 20% bleach), eggs were collected and were left to hatch for about 18 hours in M9 buffer. In the absence of food, the L1 larvae remain arrested at that stage and thus enabled starting all treatments on age-synchronized worms. Before transfer, OP50 food was spread on the control and treatment plates 15-30 minutes and were left to dry. Then, approximately equal numbers of L1 larvae were transferred to the seeded control and treatment plates. The treatment period lasted for 30 hours only at 20°C unless otherwise noted (e.g. temperature sensitive worms grown at 22°C and 25°C were exposed for 28 and 22 hours, respectively).

Reproduction assay

After treatment, worms were left to grow till late L4/young adult stage on NGM plates. Then, six centimeter NGM plates were prepared and were labeled by treatment. The plates were seeded with a 10µl drop of OP50 bacteria and left to dry. From each treatment group, four worms were transferred to their corresponding plate. Once the egg laying started, adult worms were then transferred to a new NGM plate every 24 hours, while the older plate was sealed and placed at 4°C. This allowed for easier counting as larvae were sessile and the eggs were preserved. The process was repeated for a total of two days. Four replicates were used for each treatment group with four worms per replicate and the progeny count was normalized per worm. Worms that burrowed in the agar or were lost during transfer were excluded from the analysis on the respective days.

Apoptosis assay

Nicotine treatment was only limited to the L1-L4 period, afterwards, worms were transferred to fresh NGM to grow to first day of adulthood during which the apoptosis assay was performed. To visualize the apoptotic cells, SYTO12 (Gumienny, Lambie, Hartwieg, Horvitz, & Hengartner, 1999) or acridine orange staining (Lant & Derry, 2014) were used for easier distinction of apoptotic cells in the gonad loop region (i.e., death zone) (Supplementary figure 4.1). Briefly, worms were collected and stained with ~30µM SYTO12 (Invitrogen) for 2 hours or 0.075mg/ml acridine orange for 1.5 hours. Afterwards, they are washed with M9 and transferred to a seeded NGM plate for 1.5 hours to regurgitate the dye from their intestine. Worms were then mounted on 2% agarose pads onto Zeiss inverted fluorescence microscope to count apoptotic cells in gonadal death zone. As a negative control, we used the apoptosis defective mutant (MT1522 ced-3(n717) IV) while MD701 was used as a positive control for staining.

Lifespan assay

After worms were exposed to nicotine for 30 hours, they were collected, washed with M9 buffer, and transferred to nicotine free NGM plates until late L4/early adulthood. Then, an average of 200 worms were transferred to a labelled 6 cm NGM plate seeded with OP50 from each of the control, low (20µM), and high (20mM) treatment groups, respectively. Every other day, all worms were counted and categorized as dead or alive or lost. Living worms were transferred to a new NGM plate and the process was repeated until all worms died. Worms were considered dead when they didn't move upon gentle touch and with a 5µl drop of M9 buffer. Worms that were stuck in the agar or on the plate wall were censored. Two to four trials were performed for each strain.

Data analysis

One way ANOVA was used for reproduction and apoptosis assays in SPSS (20). Post-hoc test was performed for pairwise comparisons. Bonferroni correction was applied to compute statistical differences between treatment groups at $p<0.05$. Lifespan assays were organized in excel sheets and were inputted into OASIS which is a free user-interface software for lifespan analyses (J. S. Yang et al., 2011). Kaplan Meier curves were constructed and chi square test was used to compute statistically significant differences.

Results

Post-embryonic nicotine exposure did not affect reproduction

While the low nicotine dose did not hinder worm growth, we noticed that development was delayed after exposure to the high nicotine dose (Figure 3.3A). We were interested in

checking if this delay was associated with a negative effect on reproductive success. The reproduction assay was performed as described in the methods section. A delay in development is not necessarily associated with a smaller progeny size. Thus, the assay started once the worms started laying eggs to control for the delay in development. Fertile progeny included larvae and fertile eggs after a 48 hour period. As shown in Figure 4.1A, postembryonic exposure to either low or high nicotine doses did not impact the worms' fertility [$F(2,9)=1.28$; $p=0.324$].

Post-embryonic nicotine exposure altered lifespan

We were interested in the effect of 30 hour larval nicotine exposure on the mean lifespan in adult N2 worms. Exposure to the low nicotine dose did not impact the mean lifespan [$\chi2=0.09$; $p=1$]. On the other hand, the high nicotine dose significantly decreased the mean lifespan by more than 5% relative to the control worms [$\chi2=10.26$; $p=0.0027$]. This effect was lost in the miR-80 (nDf53) loss of function mutant [$\chi2=0.24$; $p=1$] where the mean lifespan of the low and high treatment groups was similar to the control group (12 days) (Figure 4.1B). This suggests that the decrease in lifespan associated with exposure to the high nicotine dose was at least partially dependent on miR-80 and that the latter is pro-ageing.

Post-embryonic nicotine exposure increased germline apoptosis

We wanted to check how early nicotine exposure can have an enduring effect on the germ line in the worm. So, after the 30 hour larval exposure, worms were washed off treatment and transferred to NGM plates seeded with OP50 as a food source. Worms were left to grow till after egg laying had started in the first day of adulthood. Worms were then stained with either SYTO12 or acridine orange as described in the methods section. Our results showed that nicotine increased the apoptotic rate in adult worms [$F(2,147)=16.53$; $p<0.001$]. Post-hoc pairwise

comparisons revealed that both the low and high doses increased the number of apoptotic cells by 119% and 90% ($p<0.001$), respectively (Figure 4.2). To rule out possible technical biases or errors associated with staining, we used the MD701 strain with ced-1 GFP fusion protein in the sheath cells. No staining is required to visualize apoptotic germ cells in this strain; instead, apoptotic cells appear as hollow circles with a fluorescent rim. Consistent with our results in the lab strain N2 background, both nicotine doses increased the apoptotic rate in the worms [$F(2,155)=6.40$; $p=0.002$] (Figure 4.2). In Chapter III, we showed that nicotine alters the worm's feeding rate. To rule out the possibility that the observed apoptosis is not due to starvation, we starved the worms on NGM plates without OP50 throughout the 30 hour nicotine exposure period. Then, worms were transferred to seeded NGM to grow till adulthood. Interestingly, the low and high nicotine doses could no longer impact the apoptotic rate of starved worms (Figure 4.2). This suggests (i) that exposure to nicotine through feeding was a prerequisite for long term effects, and (ii) that the apoptosis phenotype was specific to nicotine. To confirm that those cells are apoptotic, we repeated the procedure using an apoptosis defective mutant ced-3 (n717) and inspected the gonadal death zone. We failed to observe any stained apoptotic cells in this background, which confirmed that early nicotine exposure had an enduring effect on the germline physiology as it increased the apoptotic rate (Figure 4.2).

Candidate molecular mediators for nicotine-induced apoptosis

The treatment and staining procedure was repeated in different genetic backgrounds. The loss of function of p53-like protein cep-3 (gk138) recovered the germline apoptotic rate to normal physiological levels (Figure 4.2). This suggests that early nicotine exposure was associated with cellular DNA damage that lasted until adulthood. Interestingly, the loss of function mutation in miR-80 (nDf53) rescued the apoptosis phenotype in response to both low

and high nicotine doses. The same effect was observed with loss of function mutations in the entire miR-58 family (nDf53 III; mir-58(n4640) IV; nDf54 X) (Figure 4.2).

In other genetic backgrounds, the recovery from the increased apoptosis was dose-dependent. For example, nicotine had a significant effect on apoptosis in let-60 (n1046) background [$F(2,294)=10.54$; $p<0.001$]. This effect was due to the low nicotine dose which increased apoptosis by 80% ($p<0.001$) (Figure 4.2). On the contrary, loss of function mutations in sir-2.1 (ok434), acr-7 (tm863), and ric-3(md158) were resistant to the low nicotine dose only and were rather sensitive to the high nicotine dose. For example, apoptosis was significantly increased in sir-2.1 (ok434) worms [$F(2,147)=17.98$; $p<0.001$] and that effect was specific to the high nicotine dose which increased the apoptotic rate by 272% relative to the control. Similarly, acr-7 (tm863) and ric-3 (md158) worms were impacted by nicotine exposure [$F(2,140)=5.99$; $p=0.003$] and [$F(2,147)=21.51$; $p<0.001$], respectively. This was due to exposure to only the high nicotine dose resulting in a 102% and 214% increase in apoptosis in acr-7 (tm863) and ric-3 (md158), respectively (Figure 4.2). Taken collectively, this suggests that different combinations of molecular factors are responsive and dependent on the nicotine dose.

Co-treatment with nicotine and U0126

The *C. elegans* mpk-1is homologous to ERK2 (MAPK1) and is the most downstream kinase in the MAPK pathway (Y. Wu & Han, 1994). We wanted to check how the inhibition of mpk-1 would impact the increase in apoptosis in response to early nicotine exposure. Each replicate was comprised of two nicotine-free NGM plates and one 20mM nicotine infused NGM plate. One batch of OP50 food was mixed and then divided to three falcon tubes. DMSO was added to one tube for the vehicle control. U0126 was diluted in DMSO and then added to the

other OP50 tubes resulting in a final concentration of 50µM (F. Chen et al., 2008). Fifteen minutes before the start of exposure, DMSO-OP50 mix was spread on NGM plates for vehicle control, U0126-OP50 mix was spread on either nicotine-free NGM, or NGM infused with 20mM of nicotine, respectively. After the plates dried, equal amounts of synchronized L1 larvae were transferred onto the plates and were left to grow for 30 hours under those conditions. Then, worms were washed off treatment and transferred to NGM free of both U0126 and nicotine and allowed to grow until adulthood. Once egg laying started, adult worms were collected, stained, and inspected as described in the methods section. Our results showed that the high nicotine dose could no longer increase the apoptotic rate in the presence of U0126 in the N2 background [$F(2,267)=2.84$; $p=0.06$] (Figure 4.3A). The same pattern was observed in miR-80 (nDf53) background with or without U0126. Interestingly, sir-2.1 (ok434) were resistant to the proapoptotic effects of the high nicotine dose when co-treated with U0126 [$F(2,267)=0.11$; $p=0.898$] (Figure 4.3A). This suggests that the 272% increase in germline apoptosis in sir-2.1 (*lof*) mutants (Figure 4.2) was due to nicotine-induced upregulation of mpk-1. From this data, we deduced that (i) nicotine induced apoptosis was due to the overactivation of mpk-1, (ii) the loss of function of sir-2.1 sensitized the worms to nicotine's proapoptotic effects and that this was due to hyperactivation of mpk-1, and (iii) mpk-1 overactivation could be a direct or a side-effect of nicotine's effect on sir-2.1.

The role of pharyngeal mpk-1 on nicotine-induced germ line apoptosis

In chapter III, we showed that nicotine overactivated mpk-1 in the pharynx. We were intrigued to see the effect of early nicotine exposure on worms with an extrachromosomal overexpression of mpk-1 GFP fusion in the pharynx. Using the same treatment and staining procedure, we inspected the number of apoptotic cells in the gonads of adult worms positive for

pharyngeal GFP. Unexpectedly, neither nicotine dose resulted in an increased apoptotic rate when mpk-1 was constitutively overexpressed in the pharynx in N2 background [F(2,136)=0.01; p=0.99] (Figure 4.3B). Worms with miR-80 loss of function were consistently not impacted with mpk-1 pharyngeal overexpression [F(1,138)=2.95; p=0.055]. On the contrary, sir-2.1 loss of function was initially sensitized to the high nicotine dose and this effect was lost when mpk-1 was constitutively expressed in the pharynx [F(1,118)=0.62; p=0.542] (Figure 4.3B). These results suggest that (i) there is cell non-autonomous signaling that links pharyngeal overexpression of mpk-1 with germ line apoptosis, (ii) mpk-1 overexpression in the pharynx protected the worms against nicotine's late onset cell death, (iii) constitutive mpk-1 overexpression in the pharynx potentially negated nicotine-induced adult germ line apoptosis either through inhibition of germ line mpk-1 aberrant activation or through mpk-1independent mechanisms, and (iv) miR-80 is a possible mediator of this protective effect or acts through a parallel mechanism.

Discussion

Early nicotine exposure impacted mean lifespan, but not reproductive success in C. elegans

In Chapter III, we showed that exposure to the high nicotine dose was associated with a developmental delay. This effect was reversible since the worms resumed growth at the normal rate when washed off of treatment onto nicotine-free treatment plates with food. Those worms progressed to sexual maturity and started laying eggs12-24 hours after the control and low treatment groups (data not shown). Despite the delayed onset, reproduction was not impacted by either nicotine dose. This is consistent with previous reports on nicotine-induced delay in conception (CDC, 2010b); however, it did not decrease fertility (Figure 4.1A). The latter was

inconsistent with previous reports linking early nicotine exposure to reduced fertility (Bruin et al., 2010; CDC, 2010b). Toxicology studies employing reproduction as a sub-lethal endpoint usually expose *C. elegans* worms between the last larval stage L4 and adulthood. Spermatogenesis starts during late L4 and oocytes differentiate in young adults. Thus during this developmental period, the number of mature germ cells is maximal which makes the reproduction assay more sensitive to assessing fertility while controlling for growth retardation side effects (Boyd, McBride, Rice, Snyder, & Freedman, 2010). Our treatment procedure ended at early L4 stage before any germ cells had differentiated which might have impacted the sensitivity to nicotine's effects on reproduction. This is also supported by the fact that *C. elegans* worms arrested at L1 develop increased stress resistance which is a feature used when freezing worm stocks for long term storage at -80°C (J. A. Lewis & Fleming, 1995). Consistent with this point, when worms were left to grow on nicotine, some worms reached sexual maturity though at a much slower pace and produced much fewer viable progeny (data not shown).

In spite of L1 resistance, this early nicotine exposure decreased mean worm lifespan by about 5% and this effect was lost in miR-80 loss of function mutant (Figure 4.1B). In *C. elegans*, the genes involved in L1 arrest and stress resistance are also implicated in adult lifespan (Baugh, 2013). Interestingly, in spite of this resistance property, early nicotine exposure still negatively impacted adult lifespan and this effect could be at least partially promoted by the overexpression of miR-80 in response to nicotine ((F. A. Taki et al., 2014; F. A. Taki et al., 2013). This is consistent with previous reports that showed that miR-80 loss of function prolonged lifespan through the regulation of the Dietary Restriction (DR) metabolic program (Vora et al., 2013). Indeed, our results also showed that miR-80 (nDf53) loss of function increased mean lifespan by 19% relative to N2 control worms.

Post-embryonic nicotine exposure increased adult germ line apoptosis

Nicotine is both pro and anti-apoptotic and this bivalent effect is context-dependent (e.g. dose, development, duration, organ) (Zeidler et al., 2007). Based on our preliminary data, exposure to both the low and high nicotine concentrations increased germ cell apoptosis in *C. elegans*. The low dose (20µM) is 1000 times lower than the high dose (20mM) yet it resulted in similar apoptotic rates (Figure 4.2). This reinforces the idea that no tobacco and nicotine exposure level is risk free (CDC, 2014; Zhou et al., 2014).

Both mpk-1 isoforms were shown to interact with cep-1 in the apoptotic pathway (Rutkowski et al., 2011). Our data showed that cep-1/p53 like loss of function rescued apoptosis in response to both doses (Figure 4.2), which suggests that it could be downstream of mpk-1 activation. cep-1/p53 activation usually reflects the occurrence of DNA damage. Therefore, early nicotine exposure impacted DNA integrity which is a trigger for cell apoptosis.

The main and direct targets of nicotine are the nicotinic and muscarinic acetylcholine receptors. *C. elegans* has around 29 nAChR subunits classified into 5 different families (Jones, Davis, Hodgkin, & Sattelle, 2007). acr-7 is a member of the acr-16 like family and is homologous to the alpha 7-10 subunit in vertebrates (Jones & Sattelle, 2004). Previously it was reported that acr-7 (*lof*) conferred resistance against the delay in development induced by 1-2mM of nicotine (Saur et al., 2013). Here, we report that acr-7 partially rescued apoptosis in response to the low nicotine dose; however, it was insufficient to rescue apoptosis in response to the high dose (Figure 4.2). Similarly, the same effect was observed with ric-3 loss of function (Figure 4.2). ric-3 is necessary for the maturation of at least 4 acetylcholine receptors and therefore its loss of function correlates with the reduction of cholinergic transmission and was reported to confer

resistance against nicotine paralysis (Halevi et al., 2002). Both acr-7 and ric-3 loss of functions could not recover apoptosis back to normal levels with the high nicotine dose. This suggests that nicotine induces apoptosis via different mechanisms depending on the dose. acr-7 and ric-3 could be part of a nicotine-specific response as was observed in response to the low dose. Meanwhile, apoptosis induced by the high nicotine dose could be a side-effect of organism level signaling.

Revisiting apoptosis in different mpk-1 backgrounds

let-60 (n1046) results in constitutive activation of Ras/MAPK yet is not associated with increased apoptosis (Gumienny et al., 1999) and that was also evident in our data as let-60 (n1046) control did not differ significantly from N2 control (Figure 4.2). let-60 (n1046) did not rescue apoptosis induced by the low nicotine dose; however, it rescued apoptosis induced by the high nicotine dose (Figure 4.2). let-60 (n1046) doesn't increase the protein levels of mpk-1, but instead alters its phosphorylation levels (M. H. Lee et al., 2007). In particular, let-60 (n1046) is associated with hyper-phosphorylation of mpk-1a isoform and hypo-phosphorylation of mpk-1b isoform. mpk-1b is the predominant isoform in the germline (M. H. Lee et al., 2007). This suggests that the low nicotine dose is associated with an increase of phosphorylation of mpk-1b in the germline resulting in increased apoptosis (Figure 4.2). Why was this effect lost in response to the high nicotine dose in let-60 (n10460) (Figure 4.2)? To answer this question, we consider the case with N2 worms with mpk-1a overexpression in the pharynx. When the same nicotine treatment procedure was applied onto these worms, apoptosis was no longer higher than control (Figure 4.2). Why does the hyperactivation of mpk-1a in the pharynx rescue the apoptosis phenotype in response to nicotine? We suggest that the increase of mpk-1a in the pharynx and body alters the feeding rate and models dietary restriction in the worm. This is supported by the lower intestinal bacterial concentration in a significant proportion of worms with mpk-1a

overexpression in the pharynx (Figure 4.4). It is important to note that this mpk-1 induced dietary restriction is not starvation as growth was not arrested and worms laid viable eggs. Dietary restriction activates many antioxidative signaling pathways that could have protected and masked nicotine-induced apoptosis. With this in mind, this could have been the case in let-60 (n1046) worms treated with the high nicotine dose which hyperactivates mpk-1a isoforms in the pharynx and body prior to the germline development and shifts global metabolism to dietary restriction mode. Worms are removed off of nicotine after 30 hours, by then dietary restriction had been established and it prevented the nicotine-induced hyperphosphorylation of mpk-1b in the germline once fully developed. This might have also been the case for exposing worms to nicotine without a food source as starvation activated mpk-1 in the pharynx (You et al., 2006). Control, low, and high groups were all arrested at L1 for the entire 30 hour period and resumed growth once washed and transferred onto OP50 seeded NGM plates. This starvation scenario made the worms resistant to nicotine's effect on apoptosis which could be through DR-like mechanisms as described above.

Are mpk-1 and sir-2.1 inversely proportional?

Since sir-2.1 loss of function enhanced nicotine-induced apoptosis and that effect was lost when nicotine was co-treated with U0126 (Figure 4.3A), then sir-2.1 (*lof*) resulted in further hyperactivation of mpk-1 which increased apoptosis. Based on this data, we would conclude that sir-2.1 and mpk-1 are inversely proportional; however, this was only observed in response to the high nicotine dose and not the low dose. As shown in Figure 4.2, sir-2.1 loss of function rescued the apoptotic effect of the low nicotine dose, so this shows that sir-2.1 and mpk-1 are positively correlated. This positive relationship is also previously reported where SIRT1 activated ERK1/2 (Y. Li et al., 2008; Renthal et al., 2009). Therefore, the hyperactivation of mpk-1 in the absence

of sir-2.1 in response to the high nicotine dose could be a side-effect of organism level response to the high dose.

ROS: a candidate signaling molecule for nicotine-induced molecular memory

Nicotine exposure started at the L1 post-embryonic stage and ended around the L3/L4 molt in *C. elegans*. Worms were then washed off treatment and allowed to grow on a nicotine free medium until sex maturation and the onset of adulthood. To recap germ line development in *C. elegans*, germ cells differentiate around the four-cell stage during embryogenesis. However, further differentiation of the somatic gonad is coupled with that of germ cells and occurs post-embryonically. Those germ cell precursors require signaling and nutrients from the developing somatic gonad for subsequent steps. The process starts in mid-L1 until L3 stage where a somatic "niche" is formed from 12 cells: 2 distal tip cells (DTCs) and 10 proximal cells. This represents the backbone for subsequent bursts in germ and somatic cell proliferation and marks the beginning of sex determination. As worms grow till L4 and young adult stages, events continue to be spatiotemporal starting from DTC-dependent germ cells mitosis which then transition to meiosis around gonad loop region. This is the site of apoptosis and is known as the death loop. Until this point, germ cells are syncytial and share a cytoplasm during the pachytene stage. This basal level of apoptosis is considered a normal physiological phenomenon that does not only eliminate faulted germ cells, but it also provides nutrients to the qualified cells through the shared cytoplasm (Hubbard & Greenstein, 2005). It is important to note that apoptosis does not always correlate with fertility, and in fact has been reported to be inversely proportion in some situations (Gartner A. et al., 2008). This was also observed in our data as nicotine increased the apoptotic rate (Figure 4.2), but did not impact fertility in this model organism (Figure 4.1A). In addition, MAPK has been shown to be a major biological hub that controls both: progression

beyond pachytene as well as the apoptosis rate. Thus, decreased MAPK signaling is proportional to pachytene arrest, decrease in apoptosis, as well as a decrease in fertility. Based on our results, we concluded that both nicotine doses increased apoptosis through different mechanisms that converged on MAPK signaling and cep-1/p53 as a likely downstream candidate to promote cell death. Our apoptosis assay is usually done on the first day of adulthood after which F1 embryos have been laid. The apoptosis assay is concerned with the loop region of the adult gonad which is comprised of both oocyte progenitor and non-progenitor cells. It is important to note that nicotine exposure preceded the differentiation of sperms or oocytes. So how come do we still see an increase in apoptosis in meiotic oocytes? Is apoptosis in this region at that time a consequence of direct nicotine exposure? One can argue that nicotine could directly have impacted the mitotic progenitor cells that migrated after nicotine was removed and eventually died. We argue that this observed apoptosis response is not due to direct nicotine exposure, but instead due to a nicotine-induced molecular memory that persisted until at least adulthood. Below, we provide a rationale for this proposition.

Did those pachytene cells in the loop arise from the meiotic cells directly exposed to nicotine that migrated to the loop during development? The answer to this question is two folds: For worms treated with the low nicotine dose, apoptosis increased in a manner similar to those exposed to the high dose (Figure 4.2). Those worms were similar to control in growth rate and reproductive onset and showed no signs of a delay. We argue that those apoptotic cells were not due to direct contact with nicotine, and that nicotine induced a stress response that was enduring. This is supported by the fact that hermaphrodite germ cells are first seen in the pachytene stage during the late-L3 stage and those cells enter meiosis to become sperm during the L4 stage. On the other hand, germ cells enter meiosis midway through L4 and later differentiate into oocytes

in adults (Kimble & Crittenden, 2007). Therefore, the only exposed meiotic cells were sperm progenitors, while the progenitors for oocytes did not coincide with nicotine exposure. For worms treated with the high nicotine dose, apoptosis also increased (Figure 4.2). Those worms were developmentally delayed. In other words, for the entire nicotine exposure period, most of the worms remained in the L1 stage as confirmed with our seam cell counts (Figure 3.3). As we recall from the timeline of germline development, the gonad has not undergone any cell division or expansion at this point. Therefore, the only exposed cells were the two germ stem cells. Taken together, this suggests that there is intragenerational inheritance of a nicotine-induced signal. What could this signal be?

ROS are great candidates for nicotine induced intragenerational signaling molecule

What make ROS *(Reactive Oxygen Species)* a highly likely candidate are their chemical properties. They bind covalently to their targets, and in most cases can be reversed. Targets include ROS-sensitive transcription factors and signaling molecules that can result in cellular and organism level responses (Ray, Huang, & Tsuji, 2012). ROS can diffuse through membranes via aquaporins and anion channels (Fisher, 2009) and mediate intercellular signaling. Sensors of ROS levels include cep-1/p53 which in addition to its canonical role as a tumor suppressor, it is also known as an antioxidant (D'Autreaux & Toledano, 2007) which can respond to ROS in a dose-dependent manner (i.e. antioxidant activation vs. apoptosis (Rutkowski et al., 2011)). ROS results in oxidation of the Cys residues on proteins and their inactivation. The hyperactivation of mpk-1 in adult germ line despite the absence of nicotine could be due to the ROS-mediated inactivation of the phosphatases (e.g. lip-1 in *C. elegans*) that inactivate the kinases as an autoregulatory feedback loop to keep signaling under control (Ray et al., 2012; Schieber & Chandel, 2014). This results in the constitutive activation of the MAPK pathway and increased

apoptosis, especially in sir-2.1 (*lof*) mutants as observed in our study. This is consistent with the role of sirtuin homologs like SIRT1in enhancing the organisms antioxidant responses (Berdichevsky, Viswanathan, Horvitz, & Guarente, 2006; Olmos et al., 2013; Tissenbaum & Guarente, 2001).

Epidemiological evidence

When considering epidemiological data concerned with mortality rates relative to smoking prevalence, some countries like Lebanon stand out. Lebanon has a significantly high level of smokers and has the highest rates among adolescents yet has one of the lower death rates in the region in the Middle East and Europe (Figure 4.5). There are around 18 religious groups in Lebanon (USDOS, 2011) and they all have one form of an annual fasting habit (Trepanowski & Bloomer, 2010). Fasting is in some ways similar to dietary restriction especially in boosting the anti-oxidant system (Trepanowski & Bloomer, 2010). This common cultural and religious habit could be the reason for the discrepancies between smoking and death rates in the country.

Applications of MS80 (mpk-1, sir-2.1, and miR-80 network)

In our study, loss of function of sir-2.1 further sensitized the worms to apoptosis in response to the high nicotine dose. Interestingly, the human homolog SIRT1 induced antioxidants that rescued smoking-induced inflammation, DNA damage and apoptosis in aortic endothelial cells and thus promoted vasoprotection (Rutkowski et al., 2011). This suggests the high nicotine dose is associated with higher levels of reactive oxygen species that could result in oxidative stress and DNA damage and eventually cellular apoptosis. This is supported by the recovery of apoptosis levels in cep-1/p53 (*lof*) worms (Figure 4.2). Protection from oxidative damage was promoted by a DR-like program triggered by mpk-1overactivation in the pharynx.

In addition, apoptosis levels went back to normal in miR-80 loss of function mutants (Figure 4.2) as well as treatment with mpk-1/ERK2 inhibitor U0126 (Figure 4.3A). miR-80 loss of function has previously been implicated with the regulation of a DR-metabolism in *C. elegans* (Vora et al., 2013). By doing so, miR-80 (*lof*) could enhance the anti-oxidant system in the worms which confers resistance against nicotine-induced enduring effects. This could be done by delivering specific anti-miR molecules into patients with smoking enhanced neurodegenerative diseases as well as other diseases with an apoptotic component. For example, smoking has been shown to increase the apoptosis rates of beta-islet cells (Bruin, Gerstein, et al., 2008; Bruin et al., 2007) as well as cardiomyocytes (L. Wang et al., 2014). Therefore, anti-miR delivery could help in treating smoking related diabetes and cardiovascular dysfunction. In addition, smoking related cancer treatments would also benefit from this drug. Cancer cells can tolerate very high levels of ROS and evade apoptosis. Providing patients with antioxidants that scavenge the ROS makes the cancer more detrimental. Meanwhile, an anti-miR molecule that shifts the body's metabolism to a DR mode would enhance the antioxidant defense system in the body such that high ROS levels would activate p53 to induce apoptosis in the cancer cells while low ROS levels in normal levels would activate p53 antioxidant property (Schieber & Chandel, 2014) which makes this approach much more selective.

Another promising application is the delivery of anti-miR 'vaccine' to neonates and adolescents in the form of transdermal patches throughout those early stages would be sufficient to confer long term resistance. This idea is supported by the observation that treatment with MAPK inhibitor only during the first 30 hours of the worms' life was sufficient to prevent nicotine-induced late-onset apoptosis. Also, Li et al where showed that sir-2.1/SIRT1 sensitized neurons to oxidative stress partly by upregulating mpk-1/ERK1/2 (Y. Li et al., 2008).

We propose a model for nicotine-induced late-onset apoptosis. Early exposure to high doses of nicotine results in high ROS levels. ROS act as signaling molecules that mediate the intragenerational inheritance of nicotine-induced damage even after growing in nicotine free environments. In turn, ROS signaling molecules activate germline mpk-1 which interacts with cep-1 to promote cellular apoptosis. With this in mind, our data bring to light potential microRNA candidate molecules for the development of preventative medicine for neurodegenerative diseases triggered by early exposure to nicotine. By targeting human microRNAs that are functionally similar to cel-miR-80 (Chapter V), we could boost a DR-metabolic and antioxidant response in patients without changes in their diets which could alleviate the enduring complications. More so, our findings could potentially explain discrepancies in long lived smokers such that their smoking habits might have been associated with a decreased appetite and reduced food intake. The latter could induce a DR-like similar response that boosts the antioxidant system and alleviate smoking-induced oxidative stress.

References

Baugh, L. R. (2013). To grow or not to grow: nutritional control of development during Caenorhabditis elegans L1 arrest. *Genetics, 194*(3), 539-555. doi: 10.1534/genetics.113.150847

Berdichevsky, A., Viswanathan, M., Horvitz, H. R., & Guarente, L. (2006). *C. elegans* SIR-2.1 interacts with 14-3-3 proteins to activate DAF-16 and extend life span. *Cell, 125*(6), 1165-1177. doi: 10.1016/j.cell.2006.04.036

Berger, F., Gage, F. H., & Vijayaraghavan, S. (1998). Nicotinic Receptor-Induced Apoptotic Cell Death of Hippocampal Progenitor Cells. *The Journal of Neuroscience, 18*(17), 6871-6881.

Boyd, W. A., McBride, S. J., Rice, J. R., Snyder, D. W., & Freedman, J. H. (2010). A high-throughput method for assessing chemical toxicity using a Caenorhabditis elegans reproduction assay. [Research Support, N.I.H., Intramural]. *Toxicol Appl Pharmacol, 245*(2), 153-159. doi: 10.1016/j.taap.2010.02.014

Bruin, J. E., Gerstein, H. C., & Holloway, A. C. (2010). Long-term consequences of fetal and neonatal nicotine exposure: a critical review. *Toxicol Sci, 116*(2), 364-374. doi: 10.1093/toxsci/kfq103

Bruin, J. E., Gerstein, H. C., Morrison, K. M., & Holloway, A. C. (2008). Increased pancreatic beta-cell apoptosis following fetal and neonatal exposure to nicotine is mediated via the mitochondria. [Research Support, Non-U.S. Gov't]. *Toxicol Sci, 103*(2), 362-370. doi: 10.1093/toxsci/kfn012

Bruin, J. E., Kellenberger, L. D., Gerstein, H. C., Morrison, K. M., & Holloway, A. C. (2007). Fetal and neonatal nicotine exposure and postnatal glucose homeostasis: identifying critical windows of exposure. *J Endocrinol, 194*(1), 171-178. doi: 10.1677/JOE-07-0050

CDC. (2010). Reproductive and Developmental EffectsHow Tobacco Smoke Causes Disease: The Biology and Behavioral Basis for Smoking-Attributable Disease: A Report of the Surgeon General.: National Center for Chronic Disease Prevention and Health Promotion (US); Office on Smoking and Health (US).Atlanta (GA): Centers for Disease Control and Prevention (US). Available at http://www.ncbi.nlm.nih.gov/books/NBK53022/.

CDC. (2014). NicotineNational Center for Chronic Disease Prevention and Health Promotion (US) Office on Smoking and Health. The Health Consequences of Smoking—50 Years of Progress: A Report of the Surgeon General. Atlanta (GA): : Centers for Disease Control and Prevention (US). Available from: http://www.ncbi.nlm.nih.gov/books/NBK294308/.

Chen, F., Mackerell, A. D., Jr., Luo, Y., & Shapiro, P. (2008). Using Caenorhabditis elegans as a model organism for evaluating extracellular signal-regulated kinase docking domain inhibitors. *J Cell Commun Signal, 2*(3-4), 81-92. doi: 10.1007/s12079-008-0034-2

Chen, R., Clifford, A., Lang, L., & Anstey, K. J. (2013). Is exposure to secondhand smoke associated with cognitive parameters of children and adolescents?--a systematic literature review. *Ann Epidemiol, 23*(10), 652-661. doi: 10.1016/j.annepidem.2013.07.001

Cuthbertson, L., & Britton, J. (2010). Passive smoking and children's health. [Editorial]. *Clin Med (Lond), 10*(2), 113-114.

D'Autreaux, B., & Toledano, M. B. (2007). ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. *Nat Rev Mol Cell Biol, 8*(10), 813-824. doi: 10.1038/nrm2256

Durazzo, T. C., Mattsson, N., & Weiner, M. W. (2014). Smoking and increased Alzheimer's disease risk: a review of potential mechanisms. *Alzheimers Dement, 10*(3 Suppl), S122-145. doi: 10.1016/j.jalz.2014.04.009

Fisher, A. B. (2009). Redox signaling across cell membranes. *Antioxid Redox Signal, 11*(6), 1349-1356. doi: 10.1089/ARS.2008.2378

Fredriksson, A., & Archer, T. (2004). Neurobehavioural deficits associated with apoptotic neurodegeneration and vulnerability for ADHD. *Neurotox Res, 6*(6), 435-456.

Gartner A., Boag P. R., & B., B. T. (2008). Germline survival and apoptosis. WormBook: The *C. elegans* Research Community, Wormbook. Retrieved from http://www.wormbook.org. doi: 10.1895/wormbook.1.145.1.

Goriounova, N. A., & Mansvelder, H. D. (2012). Short- and long-term consequences of nicotine exposure during adolescence for prefrontal cortex neuronal network function. *Cold Spring Harb Perspect Med, 2*(12), a012120. doi: 10.1101/cshperspect.a012120

Gumienny, T. L., Lambie, E., Hartwieg, E., Horvitz, H. R., & Hengartner, M. O. (1999). Genetic control of programmed cell death in the Caenorhabditis elegans hermaphrodite germline. *Development, 126*(5), 1011-1022.

Halevi, S., McKay, J., Palfreyman, M., Yassin, L., Eshel, M., Jorgensen, E., & Treinin, M. (2002). The *C. elegans* ric-3 gene is required for maturation of nicotinic acetylcholine receptors. *EMBO J, 21*(5), 1012-1020. doi: 10.1093/emboj/21.5.1012

Haugen, A. C., Schug, T. T., Collman, G., & Heindel, J. J. (2015). Evolution of DOHaD: the impact of environmental health sciences. *J Dev Orig Health Dis, 6*(2), 55-64. doi: 10.1017/S2040174414000580

Hernan, M. A., Takkouche, B., Caamano-Isorna, F., & Gestal-Otero, J. J. (2002). A meta-analysis of coffee drinking, cigarette smoking, and the risk of Parkinson's disease. [Meta-Analysis]. *Ann Neurol, 52*(3), 276-284. doi: 10.1002/ana.10277

Hubbard, E. J., & Greenstein, D. (2005). Introduction to the germ lineWormBook (2007/12/01 ed., pp. 1-4): The *C. elegans* Research Community, WormBook. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/18050415. doi: 10.1895/wormbook.1.18.1

James, W. H. (2013). Potential explanation of the reported association between maternal smoking and autism. *Environ Health Perspect, 121*(2), a42. doi: 10.1289/ehp.1206268

Jones, A. K., Davis, P., Hodgkin, J., & Sattelle, D. B. (2007). The nicotinic acetylcholine receptor gene family of the nematode Caenorhabditis elegans: an update on nomenclature. *Invert Neurosci, 7*(2), 129-131. doi: 10.1007/s10158-007-0049-z

Jones, A. K., & Sattelle, D. B. (2004). Functional genomics of the nicotinic acetylcholine receptor gene family of the nematode, Caenorhabditis elegans. [Review]. *Bioessays, 26*(1), 39-49. doi: 10.1002/bies.10377

Kimble, J., & Crittenden, S. L. (2007). Controls of germline stem cells, entry into meiosis, and the sperm/oocyte decision in Caenorhabditis elegans. [Review]. *Annu Rev Cell Dev Biol, 23*, 405-433. doi: 10.1146/annurev.cellbio.23.090506.123326

Lant, B., & Derry, W. B. (2014). Fluorescent visualization of germline apoptosis in living Caenorhabditis elegans. *Cold Spring Harb Protoc, 2014*(4), 420-427. doi: 10.1101/pdb.prot080226

Lee, M. H., Hook, B., Pan, G., Kershner, A. M., Merritt, C., Seydoux, G., . . . Kimble, J. (2007). Conserved regulation of MAP kinase expression by PUF RNA-binding proteins. *PLoS Genet, 3*(12), e233. doi: 10.1371/journal.pgen.0030233

Lewis, J. A., & Fleming, J. T. (1995). *Basic culture methods. In Caenorhabditis elegans: Modern Biological Analysis of an Organism* (Vol. 48): Academic Press, San Diego.

Li, Y., Xu, W., McBurney, M. W., & Longo, V. D. (2008). SirT1 inhibition reduces IGF-I/IRS-2/Ras/ERK1/2 signaling and protects neurons. *Cell Metab, 8*(1), 38-48. doi: 10.1016/j.cmet.2008.05.004

Machaalani, R., Waters, K. A., & Tinworth, K. D. (2005). Effects of postnatal nicotine exposure on apoptotic markers in the developing piglet brain. *Neuroscience, 132*(2), 325-333. doi: 10.1016/j.neuroscience.2004.12.039

Martins-Green, M., Adhami, N., Frankos, M., Valdez, M., Goodwin, B., Lyubovitsky, J., . . . Curras-Collazo, M. (2014). Cigarette Smoke Toxins Deposited on Surfaces: Implications for Human Health. *PLoS One, 9*(1), e86391. doi: 10.1371/journal.pone.0086391

Olmos, Y., Sanchez-Gomez, F. J., Wild, B., Garcia-Quintans, N., Cabezudo, S., Lamas, S., & Monsalve, M. (2013). SirT1 regulation of antioxidant genes is dependent on the formation of a FoxO3a/PGC-1alpha complex. [Research Support, Non-U.S. Gov't]. *Antioxid Redox Signal, 19*(13), 1507-1521. doi: 10.1089/ars.2012.4713

Ray, P. D., Huang, B. W., & Tsuji, Y. (2012). Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cell Signal, 24*(5), 981-990. doi: 10.1016/j.cellsig.2012.01.008

Renthal, W., Kumar, A., Xiao, G., Wilkinson, M., Covington, H. E., 3rd, Maze, I., . . . Nestler, E. J. (2009). Genome-wide analysis of chromatin regulation by cocaine reveals a role for sirtuins. [Research Support, N.I.H., Extramural]. *Neuron, 62*(3), 335-348. doi: 10.1016/j.neuron.2009.03.026

Rutkowski, R., Dickinson, R., Stewart, G., Craig, A., Schimpl, M., Keyse, S. M., & Gartner, A. (2011). Regulation of Caenorhabditis elegans p53/CEP-1-dependent germ cell apoptosis by Ras/MAPK signaling. [Research Support, Non-U.S. Gov't]. *PLoS Genet, 7*(8), e1002238. doi: 10.1371/journal.pgen.1002238

Saur, T., DeMarco, S. E., Ortiz, A., Sliwoski, G. R., Hao, L., Wang, X., . . . Buttner, E. A. (2013). A genome-wide RNAi screen in Caenorhabditis elegans identifies the nicotinic acetylcholine receptor subunit ACR-7 as an antipsychotic drug target. *PLoS Genet, 9*(2), e1003313. doi: 10.1371/journal.pgen.1003313

Schieber, M., & Chandel, N. S. (2014). ROS function in redox signaling and oxidative stress. *Curr Biol, 24*(10), R453-462. doi: 10.1016/j.cub.2014.03.034

Smith, A. M., Dwoskin, L. P., & Pauly, J. R. (2010). Early exposure to nicotine during critical periods of brain development: Mechanisms and consequences. *J Pediatr Biochem, 1*(2), 125-141. doi: 10.3233/JPB-2010-0012

Soule, E. K., Lipato, T., & Eissenberg, T. (2015). Waterpipe tobacco smoking: A new smoking epidemic among the young? *Curr Pulmonol Rep, 4*(4), 163-172. doi: 10.1007/s13665-015-0124-6

Taki, F., Pan, X., & Zhang, B. (2013). Nicotine Exposure Caused Significant Transgenerational Heritable Behavioral Changes in Caenorhabditis elegans. *Excli J., 12*(1611-2156), 793-806. doi: http://www.excli.de/vol12/Zhang_10092013_proof.pdf

Taki, F. A., Pan, X., Lee, M. H., & Zhang, B. (2014). Nicotine exposure and transgenerational impact: a prospective study on small regulatory microRNAs. *Sci. Rep., 4*(7513). doi: DOI:10.1038/srep07513

Taki, F. A., Pan, X., & Zhang, B. (2013). Chronic nicotine exposure systemically alters microRNA expression profiles during post-embryonic stages in Caenorhabditis elegans. *J Cell Physiol, 229*(1), 79-89. doi: 10.1002/jcp.24419

Taki, F. A., & Zhang, B. (2013). Determination of reliable reference genes for multi-generational gene expression analysis on *C. elegans* exposed to abused drug nicotine. *Psychopharmacology (Berl)*, 1-12. doi: 10.1007/s00213-013-3139-0

Tissenbaum, H. A., & Guarente, L. (2001). Increased dosage of a sir-2 gene extends lifespan in Caenorhabditis elegans. *Nature, 410*(6825), 227-230. doi: 10.1038/35065638

Trepanowski, J. F., & Bloomer, R. J. (2010). The impact of religious fasting on human health. *Nutr J, 9*, 57. doi: 10.1186/1475-2891-9-57

USDOS. (2011). Lebanon *International Religious Freedom Report*. United States Department of State. Bureau of Democracy, Human Rights and Labor. Available from http://www.state.gov/documents/organization/193107.pdf.

Vora, M., Shah, M., Ostafi, S., Onken, B., Xue, J., Ni, J. Z., . . . Driscoll, M. (2013). Deletion of microRNA-80 activates dietary restriction to extend *C. elegans* healthspan and lifespan. *PLoS Genet, 9*(8), e1003737. doi: 10.1371/journal.pgen.1003737

Wadhwa, P. D., Buss, C., Entringer, S., & Swanson, J. M. (2009). Developmental origins of health and disease: brief history of the approach and current focus on epigenetic mechanisms. *Semin Reprod Med, 27*(5), 358-368. doi: 10.1055/s-0029-1237424

Wang, L., Li, X., Zhou, Y., Shi, H., Xu, C., He, H., . . . Shan, H. (2014). Downregulation of miR-133 via MAPK/ERK signaling pathway involved in nicotine-induced cardiomyocyte apoptosis. [Research Support, Non-U.S. Gov't]. *Naunyn Schmiedebergs Arch Pharmacol, 387*(2), 197-206. doi: 10.1007/s00210-013-0929-1

Wei, H., Alberts, I., & Li, X. (2014). The apoptotic perspective of autism. *Int J Dev Neurosci, 36*, 13-18. doi: 10.1016/j.ijdevneu.2014.04.004

WHO. (2013). World Heath Statistics 2013. *World Health Organization.* Retrieved on March.19.2014, from http://www.who.int/gho/publications/world_health_statistics/EN_WHS2013_Part3.pdf?ua=1.

Wu, Y., & Han, M. (1994). Suppression of activated Let-60 ras protein defines a role of Caenorhabditis elegans Sur-1 MAP kinase in vulval differentiation. *Genes Dev, 8*(2), 147-159.

Yang, J. S., Nam, H. J., Seo, M., Han, S. K., Choi, Y., Nam, H. G., . . . Kim, S. (2011). OASIS: online application for the survival analysis of lifespan assays performed in aging research. [Research Support, Non-U.S. Gov't]. *PLoS One, 6*(8), e23525. doi: 10.1371/journal.pone.0023525

You, Y. J., Kim, J., Cobb, M., & Avery, L. (2006). Starvation activates MAP kinase through the muscarinic acetylcholine pathway in Caenorhabditis elegans pharynx. *Cell Metab, 3*(4), 237-245. doi: 10.1016/j.cmet.2006.02.012

Zeidler, R., Albermann, K., & Lang, S. (2007). Nicotine and apoptosis. [Review]. *Apoptosis, 12*(11), 1927-1943. doi: 10.1007/s10495-007-0102-8

Zhou, S., Rosenthal, D. G., Sherman, S., Zelikoff, J., Gordon, T., & Weitzman, M. (2014). Physical, behavioral, and cognitive effects of prenatal tobacco and postnatal secondhand smoke exposure. [Review]. *Curr Probl Pediatr Adolesc Health Care, 44*(8), 219-241. doi: 10.1016/j.cppeds.2014.03.007

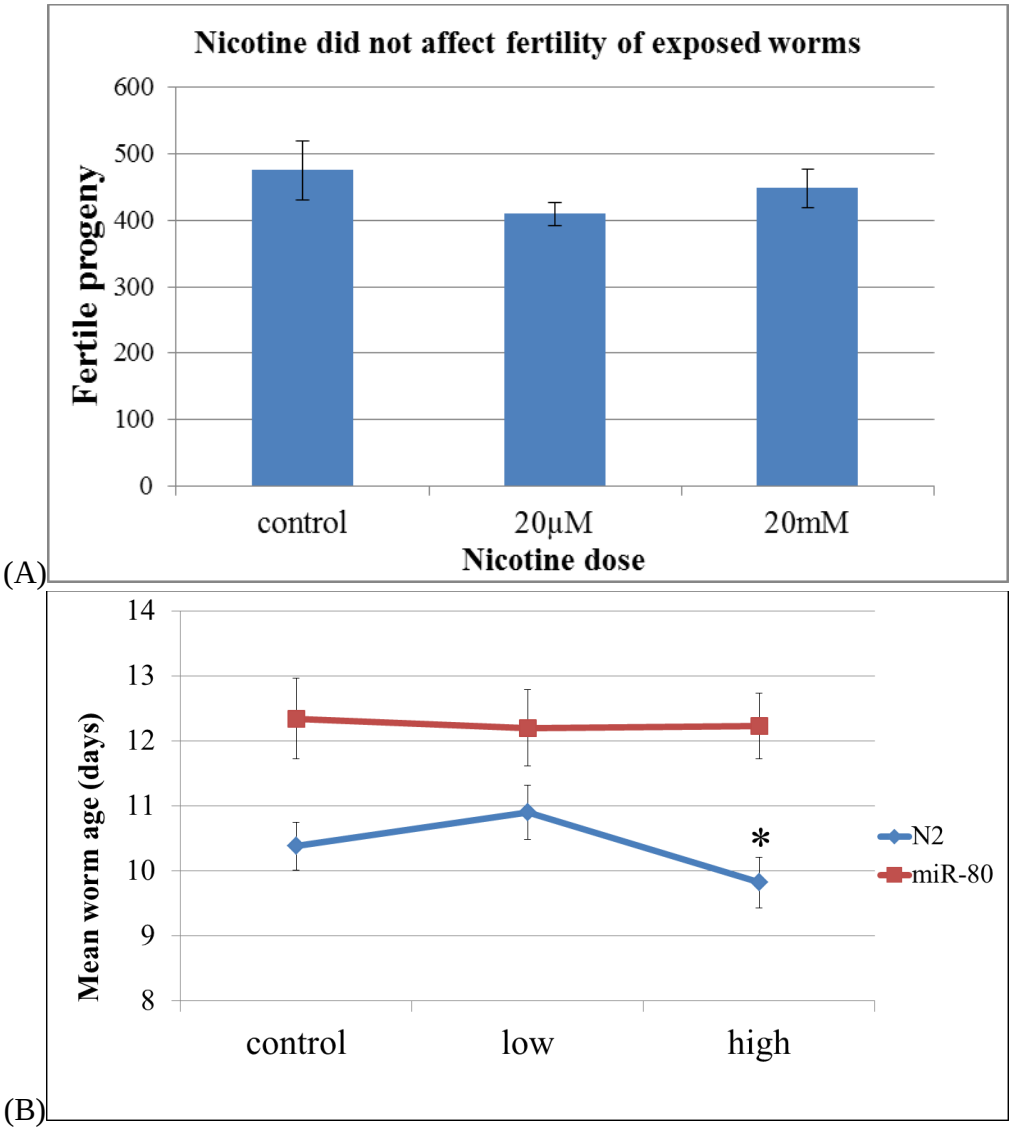


Figure 4.1: (A) The effect of postembryonic nicotine exposure on worm fertility. (B) The effect of postembryonic nicotine exposure on adult mean lifespan in N2 versus miR-80 (nDf53) (*lof*) worms. (*) denotes $p<0.05$ in comparison to the control group per strain.

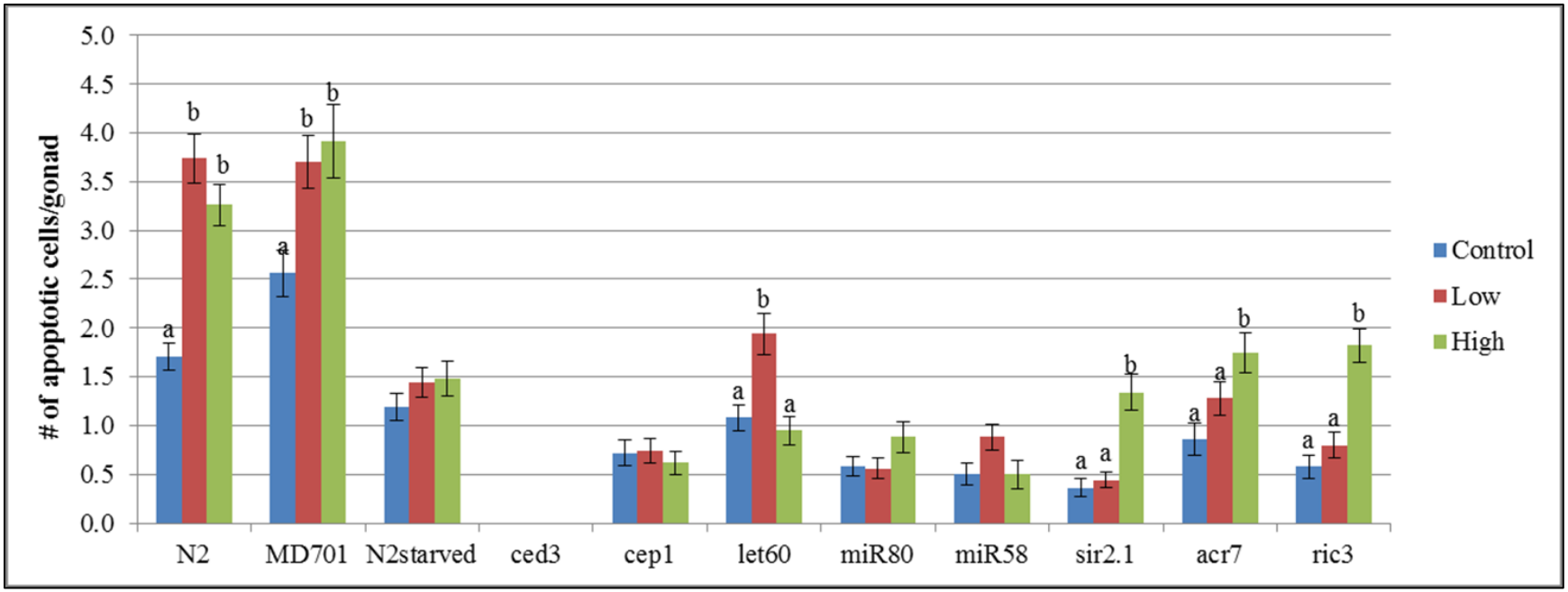
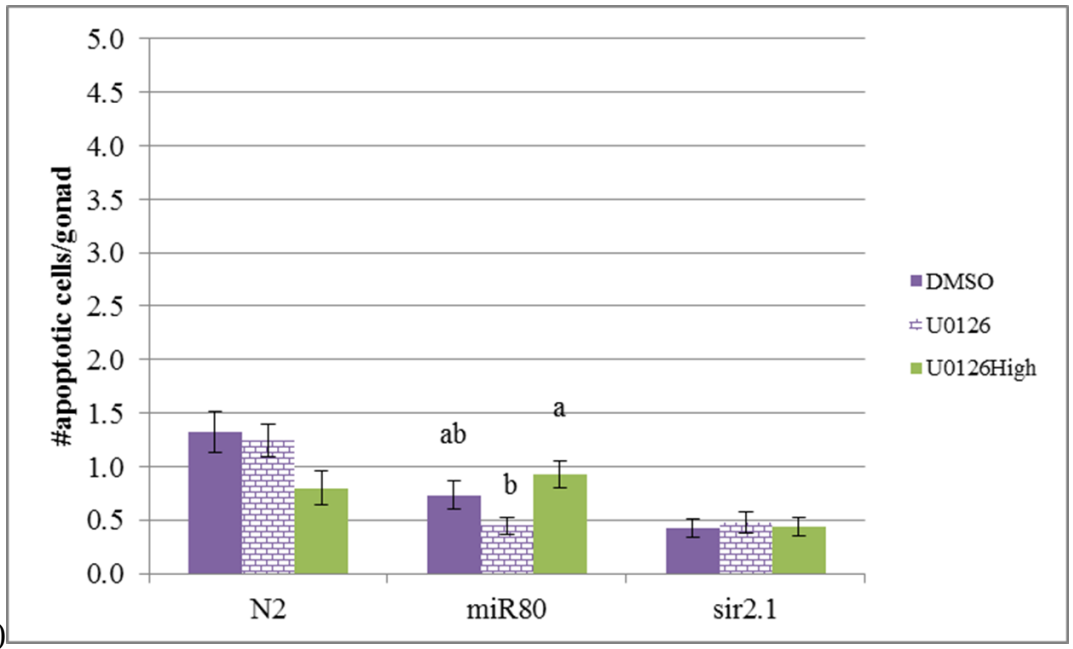


Figure 4.2: The effect of postembryonic nicotine exposure on adult germ line apoptosis in the lab strain N2 and in different genetic backgrounds (described in the text). Comparisons were done per genetic background. Differences were considered statistically significant at $p<0.05$ and are denoted by different lower case letters.

(A)



(B)

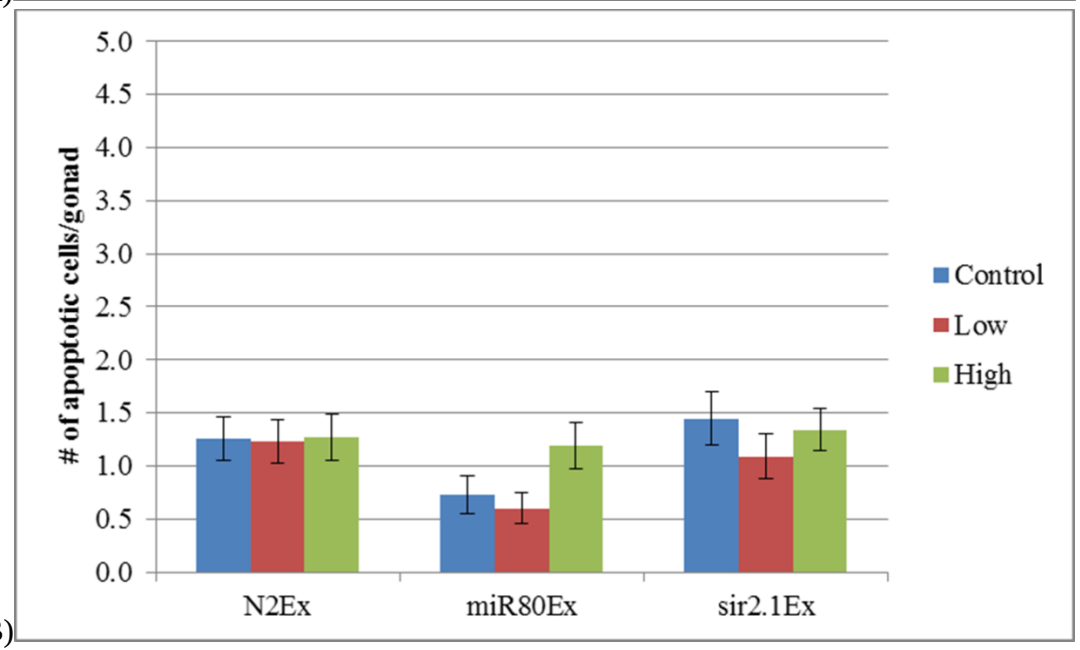


Figure 4.3: (A) The effect of U0126 co-treatment on late-onset germ line apoptosis in response to post-embryonic exposure to the high nicotine dose. (B) The effect of pharyngeal mpk-1 overexpression on germ line apoptosis in response to post-embryonic nicotine exposure.

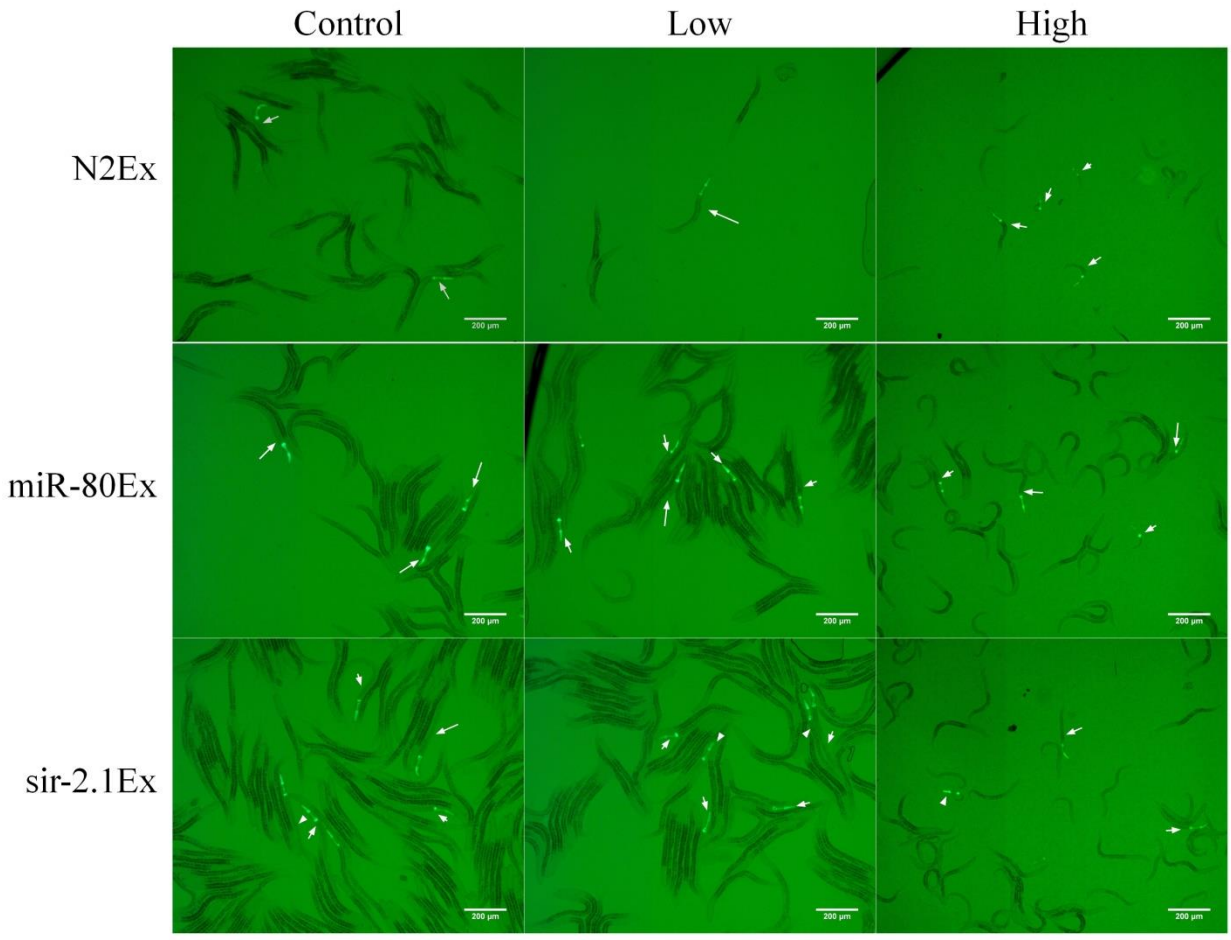


Figure 4.4: mpk-1 overexpression in the pharynx was associated with reduced intestinal bacteria in N2, miR-80 (nDf53), and sir-2.1 (ok434) backgrounds during the larval stages.

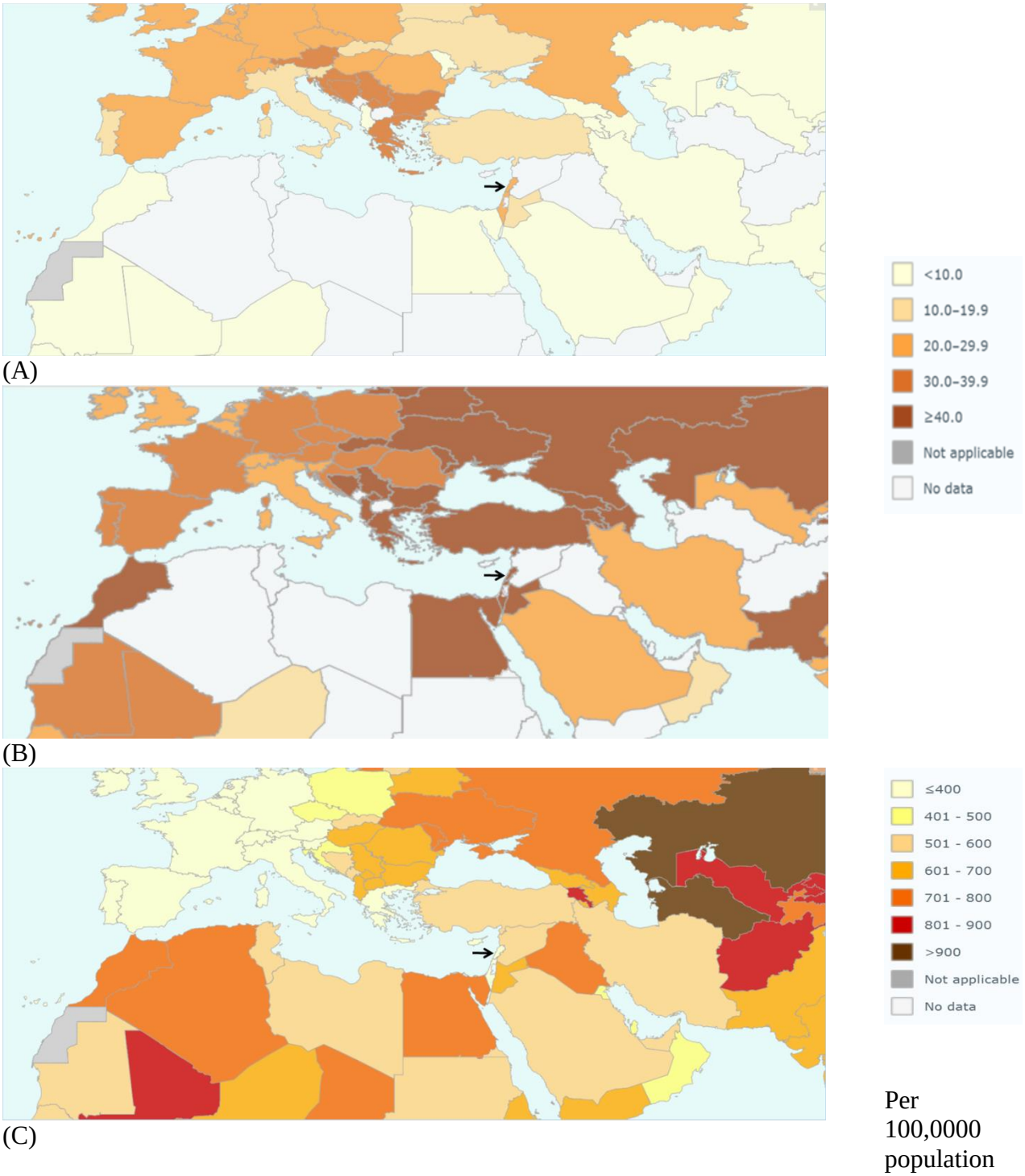
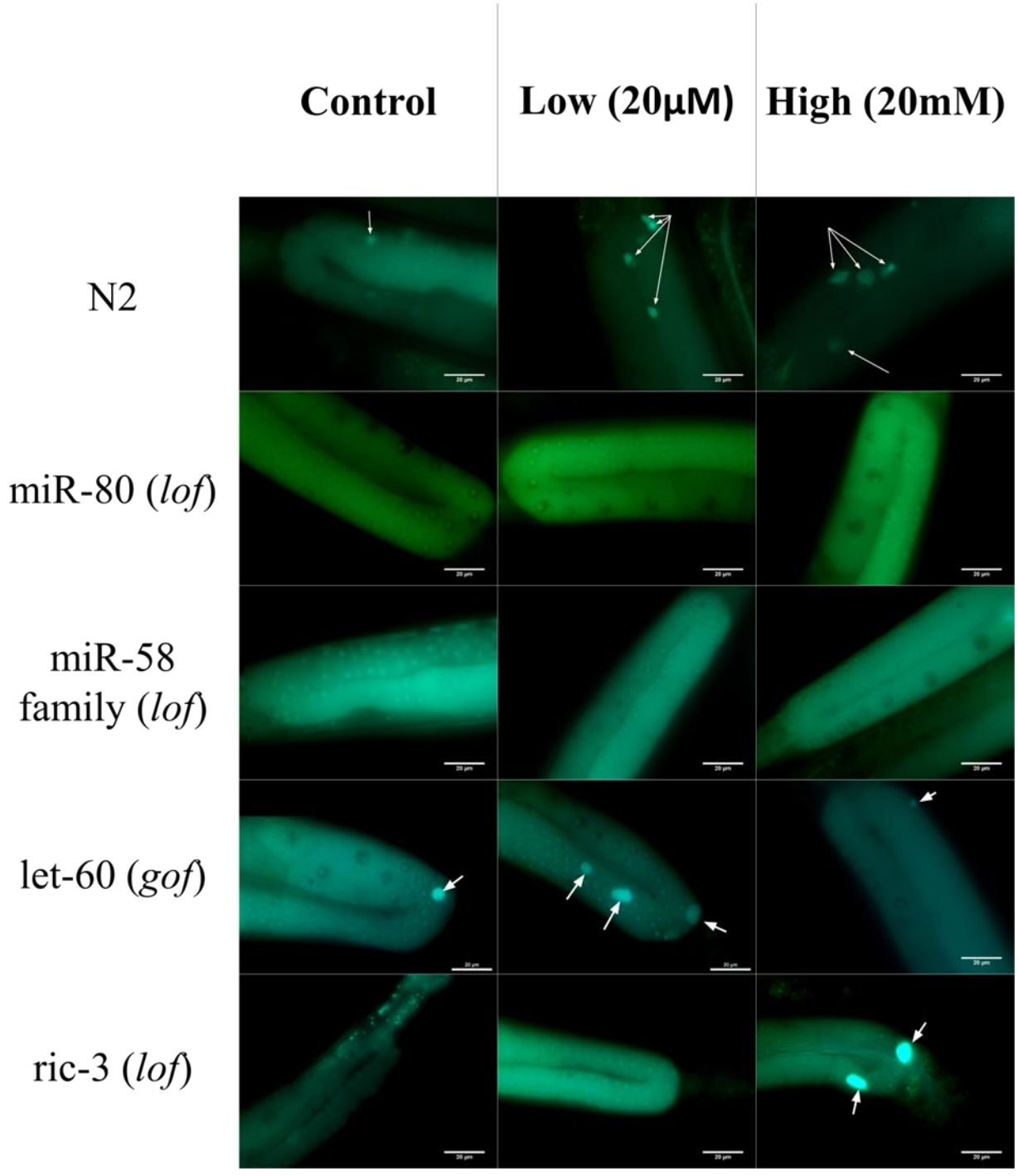


Figure 4.5: Prevalence of tobacco users among adults aged 15 or older among (A) females and (B) males in 2012 (WHO, 2015). (C) Rates of non-communicable disease (NCD) in both sexes, age standardized, per 100000 populations, 2000-2012 (WHO, 2014).

Supplementary figures



Supplementary figure 4.1: The effect was early nicotine exposure on adult germ line apoptosis was dependent on the genetic background. Arrows point to the stained apoptotic cells.

Chapter V

A hypothesized model and promising applications of nicotine-induced MS80

Abstract

Our study added more evidence to the concept of "there is no safe threshold nicotine dose below which an effect is not observed". We showed that early exposure to the high nicotine dose delayed development and the onset of reproduction, inhibited pharyngeal pumping, and decreased mean worm lifespan. Meanwhile, even the low nicotine dose during post-embryonic stage was sufficient to increase adult germ line apoptosis and that effect was similar if not slightly higher than the high dose. Our study is the first to report a possible relation among mpk-1/ERK1/2, sir-2.1/SIRT1, and miR-80 in nicotine-induced pharyngeal pumping inhibition and adult germ line apoptosis. Interestingly, we also found potential functional homologs (hsa-miR-3150a-3p, hsa-miR-3150b-3p, hsa-miR-4683, hsa-miR-4769-3p) for cel-miR-80 in humans using Clustal omega alignments and IntaRNA programs. After performing pathway enrichment analysis using DIANA-miRPath, we found that MAPK and nicotine addiction clustered together and were commonly predicted to be regulated by hsa-miR-3150b-3p. The latter could serve as cel-miR-80 counterpart in humans and an important nicotine-responsive microRNA. Based on the post-embryonic cell lineage in the worm and the exposure timeline, we suggest that the head neurons could be the major site of nicotine-induced reprogramming that could be relayed to the other tissues in the body resulting in an orchestrated and synchronized response that is triggered by ROS and mediated by MS80. Finally, we revisit the importance of epigenetic microRNA changes that precede the onset of disease which is a property that can be used as a biomarker for increased susceptibility to nicotine-induced disease in the grand-offspring generation.

Keywords:cel-miR-80, hsa-miR-3150b-3p, MAPK, nicotine addiction, transgenerational, intragenerational, sirtuins, ROS, oxidative stress, biomarkers.

MS80: A hypothesized molecular network

As mentioned earlier, the prevalence of passive and active nicotine exposure among neonates, children and adolescents remains relatively high and varies among cultures and countries. Currently, there is ample evidence that a safe nicotine dose or level does not exist. For that, early exposure to any nicotine product is considered "catastrophic" to the children's future and wellbeing (Cuthbertson & Britton, 2010). Previously, we showed that this early parental exposure altered behavior in three generations using the *C. elegans* model (F. Taki et al., 2013). Here, we showed that post-embryonic nicotine exposure in the parent generation was sufficient to induce systemic alterations in microRNA profiles till at least the grand-offspring (F2) generation (F. A. Taki et al., 2014; F. A. Taki et al., 2013). This exposure was associated with an inhibition in growth and development (Figures 3.3; 3.4) via an unidentified molecular mechanism that potentially involved aak-2/AMPK and/or CYP2A6 homologs. Based on our results, the high nicotine dose inhibited pharyngeal pumping and that effect was promoted by mpk-1 hyperactivation which was downstream of sir-2.1 and miR-80 (MS80) (Figure 3.5). Co-treatment with U0126 showed that the effect on pumping effect is not exclusively regulated by MS80, but also involved others like serotonin (Figure 3.6) and possibly aak-2/AMPK signaling. The higher sensitivity of sir-2.1 mutants to nicotine U0126 co-treatment (Figure 3.5B) suggested that sir-2.1 is an upstream transducer and results in mpk-1 hyperactivation through miR-80 dependent and independent mechanisms. Consistent with Cuthbertson and Britton's statement, this early exposure was associated with enduring effects on worm mean lifespan (Figure 4.1B) and adult germline apoptosis (Figure 4.2), but not fertility. The mean worm lifespan decreased by 5% and this effect was lost in the long lived miR-80 loss of function mutants (Figure 4.1B). This suggests that nicotine's pro-ageing effect is mediated at least partially by miR-80

upregulation. As for nicotine-induced adult germ line apoptosis, our data suggests that it is ROS-dependent upregulation of the antioxidant system via MS80, cep-1/p53, ced-3 caspase signaling. Finally, our findings showed that boosting the innate antioxidant system during early development through starvation or dietary restriction-like metabolism rescues the enduring effects of early nicotine exposure (Figures 4.3B, 4.4). Due to the conservation of MAPK and sirtuin signaling in living systems, we propose that MS80 is a promising target to 'vaccinate' children and adolescents against nicotine. Targeting this pathway would shift metabolism and enhance the innate antioxidant system and increase resistance against harmful ROS levels without risking the reduction of normal ROS signaling.

Are there cel-miR-80 functional homologs in humans?

We downloaded the sequences of all 35858 miRNAs in miRBase and looked for human and *C. elegans* miRNAs with the miR-80 six base pair sequence GAGAUC. Thirteen matches were found, five of which were for humans and were hsa-miR-3150a-3p, hsa-miR-3150b-3p, hsa-miR-4683, has-miR-4768-3p (Table 5.1). None of the human microRNAs were functionally annotated. Six of the remaining *C. elegans* microRNAs belonged to the miR-58 family as expected (Ibanez-Ventoso et al., 2008). This leaves two microRNAs cel-miR-1018 and cel-miR-1021 in which the sequence started in the fourth and fifth position, respectively (Table 5.1). The canonical microRNA seed sequence is usually from position 2 to 7 in the 5'end of the microRNA. Interestingly, a recent study used an approach called CLASH to study microRNA-target interactions and found that most of the targets did not base pair to the microRNA seed region which suggested that other sites in the microRNAs are also involved in target recognition. They added that miRNA-target interactions that depended on the seed sequence had a stronger suppressive effect than non-canonical interactions (X. Wang, 2014). This suggests that the

presence of the GAGAUC sequence in other microRNAs can also potentially contribute to the fine tuning of the targets.

Previously, miR-80 was reported to be more than 70% similar to the human homologue hsa-miR-450b-3p (Ibanez-Ventoso et al., 2008). We performed multiple sequence alignment using mature microRNA sequences for cel-miR-80-3p and the aforementioned human microRNAs hsa-miR-450b-3p, hsa-miR-3150a-3p, hsa-miR-3150b-3p, hsa-miR-4683, and hsa-miR-4768 using Clustal Omega program (Sievers et al., 2011). We confirmed that hsa-miR-450-3p was 71% similar to cel-miR-80-3p. Interestingly, cel-miR-80-3p was at least 50% similar to the remaining microRNAs: hsa-miR-4683 (57%), hsa-miR-3150a-3p and hsa-miR-3150b-3p (56%), and hsa-miR-4768-3p (53%) (Table 5.2).

Identifying potential miRNA-target interactions

We used IntaRNA (Busch, Richter, & Backofen, 2008; P. R. Wright et al., 2014) to check for possible significant interactions for sir-2.1/SIRT1 or mpk-1/MAPK1 with the mature sequences of cel-miR-80-3p, hsa-miR-450b-3p, hsa-miR-3150a-3p, hsa-miR-3150b-3p, hsa-miR-4683, hsa-miR-4769-3p. MAPK1, also known as ERK2 has more than 80% sequence identity with the *C. elegans* mpk-1 (Y. Wu & Han, 1994) and sir-2.1 is homologous to the mammalian SIRT1 (Bamps et al., 2009; Frye, 2000). IntaRNA computes an energy score that doesn't only depend on the hybridization energy, but also incorporates the accessibility of the interaction site in both RNAs as a function of their folds and lengths. We combined results from IntaRNA with the criterion of a 5' seed sequence in the 2-7 position, and found five candidate interactions for MAPK1 which could interact with hsa-miR-3150a-3p between position 1 and 11 with the proposed seed position being from 1-7 (-12.82 Kcal/mol) (Table 5.3). The second

candidate MAPK1 interaction was with hsa-miR-4768-3p along the first 1-11 bases starting with a seed positioned from the 1st to 7th base (-12.47 Kcal/mol). hsa-miR-3150b-3p was predicted to interact with MAPK1 using the bases from 1-17 with 1-7 for the seed sequence (-10.85 Kcal/mol) (Table 5.3). Similarly, MAPK1 was predicted to interact on multiple sites ranging from position 1 to 20 on hsa-miR-4683-3p starting with the seed sequence from 1-7 (-8.78 Kcal/mol). MAPK1 could also interact with hsa-miR-450b-3p from position 1 to 19 with a similar seed sequence from 1-7 (-5.17 Kcal/mol). On the other hand, fewer interactions that met our criteria were found for SIRT1. One possible interaction was with hsa-miR-3150b-3p on multiple locations along the 2nd to 19th base with the seed sequence from 2-8 (-7.62 Kcal/mol). As a side note, the lowest energy score was for a possible interaction between hsa-miR-4683 (-13.85 Kcal/mol). A lower energy correlates with a stronger interaction. The complementary base-pairing could start from the 2nd -20th position; however, the seed sequence was from 5-11 (Table 5.3). As mentioned earlier, non-canonical miRNA-target interactions are more common than originally thought and could serve a fine-tuning function rather than an extreme effect on protein levels. Our IntaRNA analysis showed a possible interaction with both MAPK1 and SIRT1 (Table 5.3). The predicted functional role for this interaction is discussed below.

Unsupervised enrichments: MAPK and nicotine addiction

We were interested in identifying the pathways enriched for those human microRNAs. DIANA-miRPath was used and was based off of microT-CDs to find microRNA-target sites in both coding and 3'UTR regions (Vlachos et al., 2012). The p-value threshold was 0.05 and the default MicroT threshold was 0.8. The program identified the pathways enriched for each miRNA and then merged them together through "Pathway Union". A summary of the results is shown as a heat map in Figure 5.2. Our initial focus was on MAPK signaling pathway (p=0.003)

which was enriched for hsa-miR-4683, hsa-miR-3051b-3p, and hsa-miR-4768. Interestingly, nicotine addiction was also enriched and was predicted to be regulated by hsa-miR-3051b-3p (p=0.007). In addition, MAPK and nicotine addiction were clustered together (Figure 5.2). Therefore, we suggest that hsa-miR-3150b-3p could be cel-miR-80 counterpart in humans and an important nicotine-responsive microRNA. Despite having the highest similarity with cel-miR-80-3p (71%) (Table 5.2), hsa-miR-450b-3p did not show enrichment for MAPK signaling (Figure 5.2). These results are encouraging and suggest a potential conserved seed sequence responsive to nicotine-induced disorders in humans.

All for one and one for all

Is there a central alarm center in the worm? Nicotine resulted in a systemic effect in the worm which was evident by the endpoints analyzed in this study: behavior, developmental arrest, and microRNA profiles. We can assume that this systemic response is triggered by a molecular alarm signal from either (i) a specific sensitive cell/tissue or (ii) multiple cells/tissue that are directly exposed. In either situation, the consequent response is synchronized and that is mainly evident in the developmental arrest.

Examples of non-autonomous signaling in *C. elegans* include HIF-1-dependent inhibition of p53-induced germ line apoptosis through the secretion of tyrosinase from ASJ head sensory neurons (Sendoel, Kohler, Fellmann, Lowe, & Hengartner, 2010). More so, larval neuronal mitochondrial perturbation was sufficient to induce the mitochondria-specific unfolded protein response (UPRmt) in the intestines. This response was maintained until adulthood and resulted in increased lifespan (Durieux, Wolff, & Dillin, 2011). The authors suggested that epigenetics modifications mediated this enduring effect to ensure resistance against future mitochondrial

stress (Durieux et al., 2011). In addition, tissue-specific rescue of microRNA biogenesis machinery (PASH-1) showed that miRNAs promote longevity in cell autonomous and non-autonomous mechanisms in *C. elegans*. So, microRNAs might not necessarily be signaling molecules by themselves, but they regulate gene expression in a cell and consequently alter its function. In turn, intra and extracellular signaling is impacted as a side-effect (Lehrbach et al., 2012). That was the case with miR-71 neuronal regulation which resulted in increased lifespan through endocrine daf-16/FOXO signaling in the intestine (Boulias & Horvitz, 2012). In addition, by using tissue-restricted RNAi and aversion response as an endpoint, Melo and Ruvkun showed that neuroendocrine signals could be relayed between neuronal and non-neuronal peripheral tissue as a result of the sensation and response to cellular exposure and stress (Melo & Ruvkun, 2012). Their findings supported the DAMP (damage-associated molecular pattern) hypothesis which involves the cellular recognition of small molecules released into the extracellular environment following pathogen-induced cell damage and lysis of their neighbors (Matzinger, 1994; Melo & Ruvkun, 2012).

Interestingly, we mentioned in chapter I that extracellular miRNAs could be carried in exosomes, apoptotic bodies, RNA binding proteins, and lipoproteins (Boon & Vickers, 2013; H. J. Lee, 2014; Rayner & Hennessy, 2013) and that this is not limited to leakage from a damaged cell, but it can actively be excreted (Bala et al., 2012). For example, neuronal PDGF-B regulation required an exosome-dependent shuttling of miR-29b from astrocytes (G. Hu et al., 2012). Thus, miRNAs can mediate organism-level signaling to synchronize communications and processes. Intercellular miRNA circulation has not been documented in *C. elegans*; however, in vitro expression of *C. elegans* cel-miR-67 in rat gliosarcoma cells was associated with intercellular transfer through gap junctions (Katakowski, Buller, Wang, Rogers, & Chopp, 2010). Those

microRNAs can also mediate signaling indirectly by influencing the intracellular ROS levels (Magenta, Greco, Gaetano, & Martelli, 2013). Indeed, ROS are required for normal signaling. For example, ROS are required for the activation of RTK/MAPK signaling to mediate normal cellular function (Finkel, 2011). An increase in ROS levels can also activate the redox-sensitive aak-2/AMPK which activates sir-2.1/SIRT1 (Salminen, Kaarniranta, & Kauppinen, 2013) and alter cell signaling and gene expression to enhance oxidative resistance (Brunet et al., 2004) or induce cellular apoptosis (Jin et al., 2007; Y. Li et al., 2008). The two main sources of ROS are membrane-bound enzymes that rely on NAPDH oxidases activity and mitochondrial cytochrome complexes (I, III) which release electrons from NADH and FADH2. Electrons can then interact with oxygen and give rise to superoxide anions (Finkel, 2011). miRNAs can act as antioxidants by activating antioxidant enzymes like GSH directly (J. M. Wang et al., 2014) or indirectly (Howell et al., 2013) or by suppressing ROS-producing enzymes like NADPH (Varga et al., 2013). Meanwhile, they can also act as pro-oxidants by increasing ROS-producing enzymes (Oenarto et al., 2016; Shilo, Roy, Khanna, & Sen, 2008). Nicotine is notorious for increasing ROS levels in mesencephalic cells (Barr et al., 2007), offspring arteries (Xiao, Huang, Yang, & Zhang, 2011), fetal and neonatal pancreatic cells (Bruin, Petre, et al., 2008), and therefore this could be mediated in part by influencing the expression levels of microRNAs.

With this in mind, we propose that nicotine causes intracellular damage. The latter includes elevated ROS levels which might be released from cells directly exposed to nicotine: neurons and the intestines (Durieux et al., 2011) (Figure 5.1). Neurons like ASG secrete a signal that results in a synchronized organism level response (i.e. developmental arrest). Worms exposed to the high nicotine dose were arrested at L1 yet had an increased germline apoptosis rate in adults even in the absence of nicotine. Once off of nicotine, worms proceeded to grow till

adulthood. This growth is associated with a 350% increase in neuronal cells in the lateral hypodermis, 170% increase in neuronal cells of the ventral cord, 70% increase in intestinal cells, and only a 2% increase in neuronal cells of the head (Figure 5.3) (J. E. Sulston & Horvitz, 1977). The increase in all the neuronal cells is contributed by the postembryonic lineage, while the increase in the intestinal cells is due to a single duplication of about 70% of the cells present at L1 (J. E. Sulston & Horvitz, 1977). The intragenerational inheritance of ROS induced by direct nicotine exposure is unlikely to be in neurons of the lateral hypodermis or ventral cord as it is more likely to be diluted due to the dramatic increase in cell count. The intestine also duplicates but for a lesser extent, so ROS remnants may, but are less likely to, result in an enduring effect. The most noticeable tissue was the head region where the neuronal cell count almost remains the same from L1 till adulthood (Figure 5.3). On a separate note, worms exposed to the low nicotine dose did not arrest at L1 and yet also had an increase in the apoptotic rate in their adult germlines. The neural head count is common to L1, L3, L4, and adult worms and therefore could be the site of enduring ROS-induced damage or signaling. This suggests that ROS in head neurons could trigger an oxidative stress response that is communicated to the remaining body tissue via miR-80 and endocrine signaling as the worms grow.

Promising potential for MS80

If the long term effects of early nicotine exposure are mediated by ROS, then would not antioxidant supplements be sufficient? The answer to this argument is two folds: (i) ROS is a signaling molecule critical for normal physiological processes and molecular signaling and a reduction in ROS below normal levels could disrupt the homeostasis and result in toxicity, and (ii) delivery of antioxidants to the brain is hindered by the blood brain barrier (Chege & McColl, 2014).

In addition, there is some evidence about the temporal sequences of epigenetics factors that suggests that it precedes the onset of the disease and can in turn be influenced by the disease itself (Relton & Davey Smith, 2012). With this in mind, we showed that miR-80 could serve as a transgenerational biomarker for early parental nicotine exposure and we showed its roles in mediating nicotine-induced early and late-onset diseases in the worm. This could imply that the upregulation of miR-80 in the grand-offspring generation increases the susceptibility of those individuals to the enduring effects such as cell apoptosis and therefore predisposes them to diseases with an apoptotic component.

References

Bala, S., Petrasek, J., Mundkur, S., Catalano, D., Levin, I., Ward, J., . . . Szabo, G. (2012). Circulating microRNAs in exosomes indicate hepatocyte injury and inflammation in alcoholic, drug-induced, and inflammatory liver diseases. [Research Support, N.I.H., Extramural]. *Hepatology, 56*(5), 1946-1957. doi: 10.1002/hep.25873

Bamps, S., Wirtz, J., Savory, F. R., Lake, D., & Hope, I. A. (2009). The Caenorhabditis elegans sirtuin gene, sir-2.1, is widely expressed and induced upon caloric restriction. [Research Support, Non-U.S. Gov't]. *Mech Ageing Dev, 130*(11-12), 762-770. doi: 10.1016/j.mad.2009.10.001

Barr, J., Sharma, C. S., Sarkar, S., Wise, K., Dong, L., Periyakaruppan, A., & Ramesh, G. T. (2007). Nicotine induces oxidative stress and activates nuclear transcription factor kappa B in rat mesencephalic cells. *Mol Cell Biochem, 297*(1-2), 93-99. doi: 10.1007/s11010-006-9333-1

Boon, R. A., & Vickers, K. C. (2013). Intercellular transport of microRNAs. *Arterioscler Thromb Vasc Biol, 33*(2), 186-192. doi: 10.1161/ATVBAHA.112.300139

Boulias, K., & Horvitz, H. R. (2012). The *C. elegans* microRNA mir-71 acts in neurons to promote germline-mediated longevity through regulation of DAF-16/FOXO. [Research Support, Non-U.S. Gov't]. *Cell Metab, 15*(4), 439-450. doi: 10.1016/j.cmet.2012.02.014

Bruin, J. E., Petre, M. A., Lehman, M. A., Raha, S., Gerstein, H. C., Morrison, K. M., & Holloway, A. C. (2008). Maternal nicotine exposure increases oxidative stress in the offspring. [Research Support, Non-U.S. Gov't]. *Free Radic Biol Med, 44*(11), 1919-1925. doi: 10.1016/j.freeradbiomed.2008.02.010

Brunet, A., Sweeney, L. B., Sturgill, J. F., Chua, K. F., Greer, P. L., Lin, Y., . . . Greenberg, M. E. (2004). Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. *Science, 303*(5666), 2011-2015. doi: 10.1126/science.1094637

Busch, A., Richter, A. S., & Backofen, R. (2008). IntaRNA: efficient prediction of bacterial sRNA targets incorporating target site accessibility and seed regions. [Research Support, Non-U.S. Gov't]. *Bioinformatics, 24*(24), 2849-2856. doi: 10.1093/bioinformatics/btn544

Chege, P. M., & McColl, G. (2014). Caenorhabditis elegans: a model to investigate oxidative stress and metal dyshomeostasis in Parkinson's disease. [Review]. *Front Aging Neurosci, 6*, 89. doi: 10.3389/fnagi.2014.00089

Cuthbertson, L., & Britton, J. (2010). Passive smoking and children's health. [Editorial]. *Clin Med (Lond), 10*(2), 113-114.

Durieux, J., Wolff, S., & Dillin, A. (2011). The cell-non-autonomous nature of electron transport chain-mediated longevity. *Cell, 144*(1), 79-91. doi: 10.1016/j.cell.2010.12.016

Finkel, T. (2011). Signal transduction by reactive oxygen species. *J Cell Biol, 194*(1), 7-15. doi: 10.1083/jcb.201102095

Frye, R. A. (2000). Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins. *Biochem Biophys Res Commun, 273*(2), 793-798. doi: 10.1006/bbrc.2000.3000

Howell, J. C., Chun, E., Farrell, A. N., Hur, E. Y., Caroti, C. M., Iuvone, P. M., & Haque, R. (2013). Global microRNA expression profiling: curcumin (diferuloylmethane) alters oxidative stress-responsive microRNAs in human ARPE-19 cells. *Mol Vis, 19*, 544-560.

Hu, G., Yao, H., Chaudhuri, A. D., Duan, M., Yelamanchili, S. V., Wen, H., . . . Buch, S. (2012). Exosome-mediated shuttling of microRNA-29 regulates HIV Tat and morphine-mediated neuronal dysfunction. [Research Support, N.I.H., Extramural]. *Cell Death Dis, 3*, e381. doi: 10.1038/cddis.2012.114

Ibanez-Ventoso, C., Vora, M., & Driscoll, M. (2008). Sequence relationships among *C. elegans*, D. melanogaster and human microRNAs highlight the extensive conservation of microRNAs in biology. [Research Support, N.I.H., Extramural]. *PLoS One, 3*(7), e2818. doi: 10.1371/journal.pone.0002818

Jin, Q., Yan, T., Ge, X., Sun, C., Shi, X., & Zhai, Q. (2007). Cytoplasm-localized SIRT1 enhances apoptosis. [Research Support, Non-U.S. Gov't]. *J Cell Physiol, 213*(1), 88-97. doi: 10.1002/jcp.21091

Katakowski, M., Buller, B., Wang, X., Rogers, T., & Chopp, M. (2010). Functional microRNA is transferred between glioma cells. *Cancer Res, 70*(21), 8259-8263. doi: 10.1158/0008-5472.CAN-10-0604

Lee, H. J. (2014). Additional stories of microRNAs. *Exp Biol Med (Maywood)*. doi: 10.1177/1535370214544269

Lehrbach, N. J., Castro, C., Murfitt, K. J., Abreu-Goodger, C., Griffin, J. L., & Miska, E. A. (2012). Post-developmental microRNA expression is required for normal physiology, and regulates aging in parallel to insulin/IGF-1 signaling in *C. elegans*. [Research Support, Non-U.S. Gov't]. *RNA, 18*(12), 2220-2235. doi: 10.1261/rna.035402.112

Li, Y., Xu, W., McBurney, M. W., & Longo, V. D. (2008). SirT1 inhibition reduces IGF-I/IRS-2/Ras/ERK1/2 signaling and protects neurons. *Cell Metab, 8*(1), 38-48. doi: 10.1016/j.cmet.2008.05.004

Magenta, A., Greco, S., Gaetano, C., & Martelli, F. (2013). Oxidative stress and microRNAs in vascular diseases. *Int J Mol Sci, 14*(9), 17319-17346. doi: 10.3390/ijms140917319

Matzinger, P. (1994). Tolerance, danger, and the extended family. [Review]. *Annu Rev Immunol, 12*, 991-1045. doi: 10.1146/annurev.iy.12.040194.005015

Melo, J. A., & Ruvkun, G. (2012). Inactivation of conserved *C. elegans* genes engages pathogen- and xenobiotic-associated defenses. *Cell, 149*(2), 452-466. doi: 10.1016/j.cell.2012.02.050

Oenarto, J., Karababa, A., Castoldi, M., Bidmon, H. J., Gorg, B., & Haussinger, D. (2016). Ammonia-induced miRNA expression changes in cultured rat astrocytes. *Sci Rep, 6*, 18493. doi: 10.1038/srep18493

Rayner, K. J., & Hennessy, E. J. (2013). Extracellular communication via microRNA: lipid particles have a new message. [Review]. *J Lipid Res, 54*(5), 1174-1181. doi: 10.1194/jlr.R034991

Relton, C. L., & Davey Smith, G. (2012). Is epidemiology ready for epigenetics? *Int J Epidemiol, 41*(1), 5-9. doi: 10.1093/ije/dys006

Salminen, A., Kaarniranta, K., & Kauppinen, A. (2013). Crosstalk between Oxidative Stress and SIRT1: Impact on the Aging Process. *Int J Mol Sci, 14*(2), 3834-3859. doi: 10.3390/ijms14023834

Sendoel, A., Kohler, I., Fellmann, C., Lowe, S. W., & Hengartner, M. O. (2010). HIF-1 antagonizes p53-mediated apoptosis through a secreted neuronal tyrosinase. *Nature, 465*(7298), 577-583. doi: 10.1038/nature09141

Shilo, S., Roy, S., Khanna, S., & Sen, C. K. (2008). Evidence for the involvement of miRNA in redox regulated angiogenic response of human microvascular endothelial cells. [Research Support, N.I.H., Extramural]. *Arterioscler Thromb Vasc Biol, 28*(3), 471-477. doi: 10.1161/ATVBAHA.107.160655

Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., Li, W., . . . Higgins, D. G. (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. [Research Support, Non-U.S. Gov't]. *Mol Syst Biol, 7*, 539. doi: 10.1038/msb.2011.75

Sulston, J. E., & Horvitz, H. R. (1977). Post-embryonic cell lineages of the nematode, Caenorhabditis elegans. *Dev Biol, 56*(1), 110-156. doi: 0012-1606(77)90158-0 [pii]

Sulston, J. E., Schierenberg, E., White, J. G., & Thomson, J. N. (1983). The embryonic cell lineage of the nematode Caenorhabditis elegans. *Dev Biol, 100*(1), 64-119.

Taki, F., Pan, X., & Zhang, B. (2013). Nicotine Exposure Caused Significant Transgenerational Heritable Behavioral Changes in Caenorhabditis elegans. *Excli J., 12*(1611-2156), 793-806. doi: http://www.excli.de/vol12/Zhang_10092013_proof.pdf

Taki, F. A., Pan, X., Lee, M.-H., & Zhang, B. (2014). Nicotine exposure and transgenerational impact: a prospective study on small regulatory microRNAs. *Sci. Rep., 4*(7513). doi: DOI:10.1038/srep07513

Taki, F. A., Pan, X., & Zhang, B. (2013). Chronic nicotine exposure systemically alters microRNA expression profiles during post-embryonic stages in Caenorhabditis elegans. *J Cell Physiol, 229*(1), 79-89. doi: 10.1002/jcp.24419

Varga, Z. V., Kupai, K., Szucs, G., Gaspar, R., Paloczi, J., Farago, N., . . . Csont, T. (2013). MicroRNA-25-dependent up-regulation of NADPH oxidase 4 (NOX4) mediates hypercholesterolemia-induced oxidative/nitrative stress and subsequent dysfunction in the heart. [Research Support, Non-U.S. Gov't]. *J Mol Cell Cardiol, 62*, 111-121. doi: 10.1016/j.yjmcc.2013.05.009

Vlachos, I. S., Kostoulas, N., Vergoulis, T., Georgakilas, G., Reczko, M., Maragkakis, M., . . . Hatzigeorgiou, A. G. (2012). DIANA miRPath v.2.0: investigating the combinatorial effect of microRNAs in pathways. [Research Support, Non-U.S. Gov't]. *Nucleic Acids Res, 40*(Web Server issue), W498-504. doi: 10.1093/nar/gks494

Wang, J. M., Tao, J., Chen, D. D., Cai, J. J., Irani, K., Wang, Q., . . . Chen, A. F. (2014). MicroRNA miR-27b rescues bone marrow-derived angiogenic cell function and accelerates wound healing in type 2 diabetes mellitus. *Arterioscler Thromb Vasc Biol, 34*(1), 99-109. doi: 10.1161/ATVBAHA.113.302104

Wang, X. (2014). Composition of seed sequence is a major determinant of microRNA targeting patterns. [Research Support, N.I.H., Extramural]. *Bioinformatics, 30*(10), 1377-1383. doi: 10.1093/bioinformatics/btu045

Wright, P. R., Georg, J., Mann, M., Sorescu, D. A., Richter, A. S., Lott, S., . . . Backofen, R. (2014). CopraRNA and IntaRNA: predicting small RNA targets, networks and interaction domains. [Research Support, Non-U.S. Gov't]. *Nucleic Acids Res, 42*(Web Server issue), W119-123. doi: 10.1093/nar/gku359

Wu, Y., & Han, M. (1994). Suppression of activated Let-60 ras protein defines a role of Caenorhabditis elegans Sur-1 MAP kinase in vulval differentiation. *Genes Dev, 8*(2), 147-159.

Xiao, D., Huang, X., Yang, S., & Zhang, L. (2011). Antenatal nicotine induces heightened oxidative stress and vascular dysfunction in rat offspring. *Br J Pharmacol, 164*(5), 1400-1409. doi: 10.1111/j.1476-5381.2011.01437.x

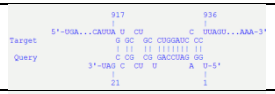
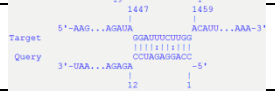
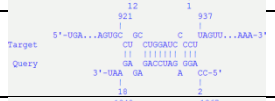
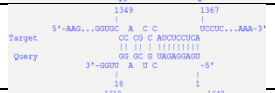
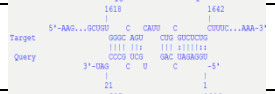
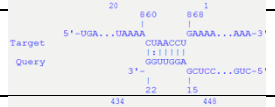
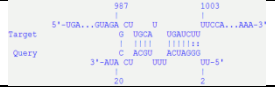
Table 5.1: *C. elegans* and humans microRNAs with GAGAUC sequence.

Position	microRNA	5'->3' mature sequence
2	cel-miR-58a-3p	UGAGAUCGUUCAGUACGGCAAU
2	cel-miR-80-3p	UGAGAUCAUUAGUUGAAAGCCGA
2	cel-miR-81-3p	UGAGAUCAUCGUGAAAGCUAGU
2	cel-miR-82-3p	UGAGAUCAUCGUGAAAGCCAGU
4	cel-miR-1018	AGAGAGAUCAUUGGACUUACAG
5	cel-miR-1021	AAGUGAGAUCAUGUGAAAUCCUCGG
2	cel-miR-58b-3p	AGAGAUCAACCAUUGAGAUCCAA
2	cel-miR-2209a-3p	AGAGAUCAGCGGUUACACUACA
6	hsa-miR-3150a-3p	CUGGGGAGAUCCUCGAGGUUGG
5	hsa-miR-3150b-3p	UGAGGAGAUCGUCGAGGUUGG
3	hsa-miR-4683	UGGAGAUCCAGUGCUCGCCCGAU
5	hsa-miR-4768-3p	CCAGGAGAUCCAGAGAGAAU
3	hsa-miR-450b-3p	UUGGGAUCAUUUUGCAUCCAUA

Table 5.2: Homology between cel-miR-80-3p and five human miRNAs based on Clustal omega.

Percent Identity Matrix (Clustal2.1)	**cel-miR-80-3p**	**hsa-miR-450b-3p**	**hsa-miR-3150a-3p**	**hsa-miR-3150b-3p**	**hsa-miR-4683**	**hsa-miR-4768-3p**
cel-miR-80-3p	100	71.43	55.56	55.56	57.14	52.94
hsa-miR-450b-3p	71.43	100	38.89	38.89	47.62	35.29
hsa-miR-3150a-3p	55.56	38.89	100	90.48	66.67	55
hsa-miR-3150b-3p	55.56	38.89	90.48	100	61.11	55
hsa-miR-4683	57.14	47.62	66.67	61.11	100	61.11
hsa-miR-4768-3p	52.94	35.29	55	55	61.11	100

Table 5.3: Predicted microRNA-target interaction using IntaRNA (Busch et al., 2008; P. R. Wright et al., 2014).

Target	Position	Query	Position	(Kcal/mol)	Seed	GU seed	GU out	Interaction
SIRT1	918 -- 935	hsa-miR-4683	2 -- 20	-13.8472	5 to 11	0	0	
MAPK1	1189 -- 1206	hsa-miR-3150a-3p	1 -- 18	-12.8174	1 to 7	1	1	
MAPK1	1448 -- 1458	hsa-miR-4768-3p	1 -- 11	-12.468	1 to 7	2	0	
SIRT1	922 -- 936	hsa-miR-4768-3p	3 -- 17	-11.4264	7 to 13	0	0	
MAPK1	1350 -- 1366	hsa-miR-3150b-3p	1 -- 17	-10.8541	1 to 7	0	0	
MAPK1	1619 -- 1641	hsa-miR-4683	1 -- 20	-8.78021	1 to 7	3	1	
SIRT1	986 -- 1005	hsa-miR-3150b-3p	2 -- 19	-7.61717	2 to 8	3	2	
SIRT1	861 -- 867	hsa-miR-3150a-3p	16 -- 22	-6.58482	6 to 22	1	0	
MAPK1	435 -- 447	hsa-miR-450b-3p	1 -- 19	-5.16529	1 to 7	0	0	
SIRT1	988 -- 1002	hsa-miR-450b-3p	3 -- 19	-4.94567	3 to 9	2	0	

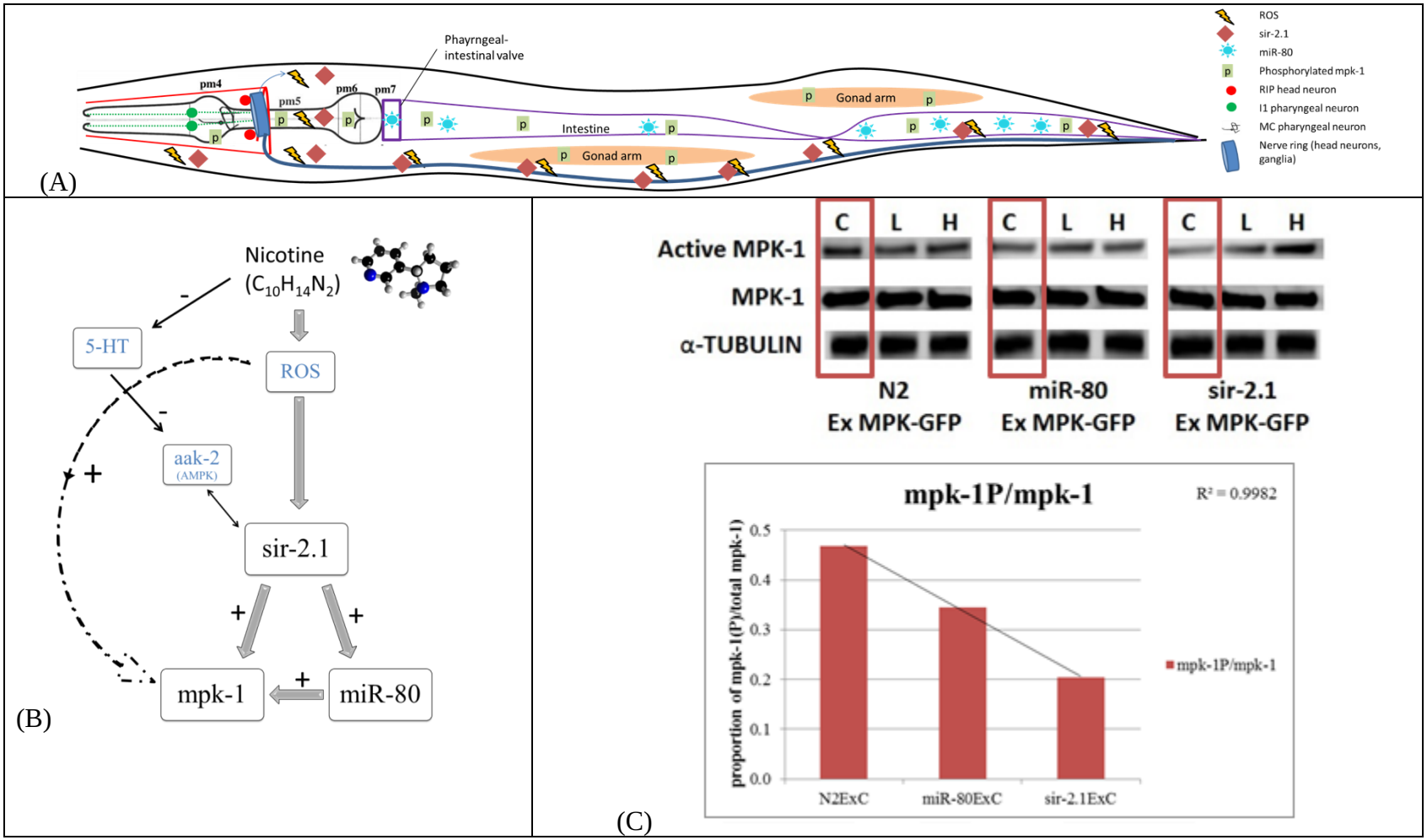


Figure 5.1: Hypothesized model for MS80 in response to postembryonic nicotine exposure. (A) The hypothesized tissue distribution of MS80 in response to nicotine based on previous reports. (B) The hypothesized sequential roles of MS80 in nicotine-induced disorders. (C) Western blots to

detect the activated mpk-1/ERK (phosphorylated) relative to total mpk-1 (You et al., 2006) in Ex myo-2 mpk-1 backgrounds. ImageJ was used for relative quantification of activated mpk-1.

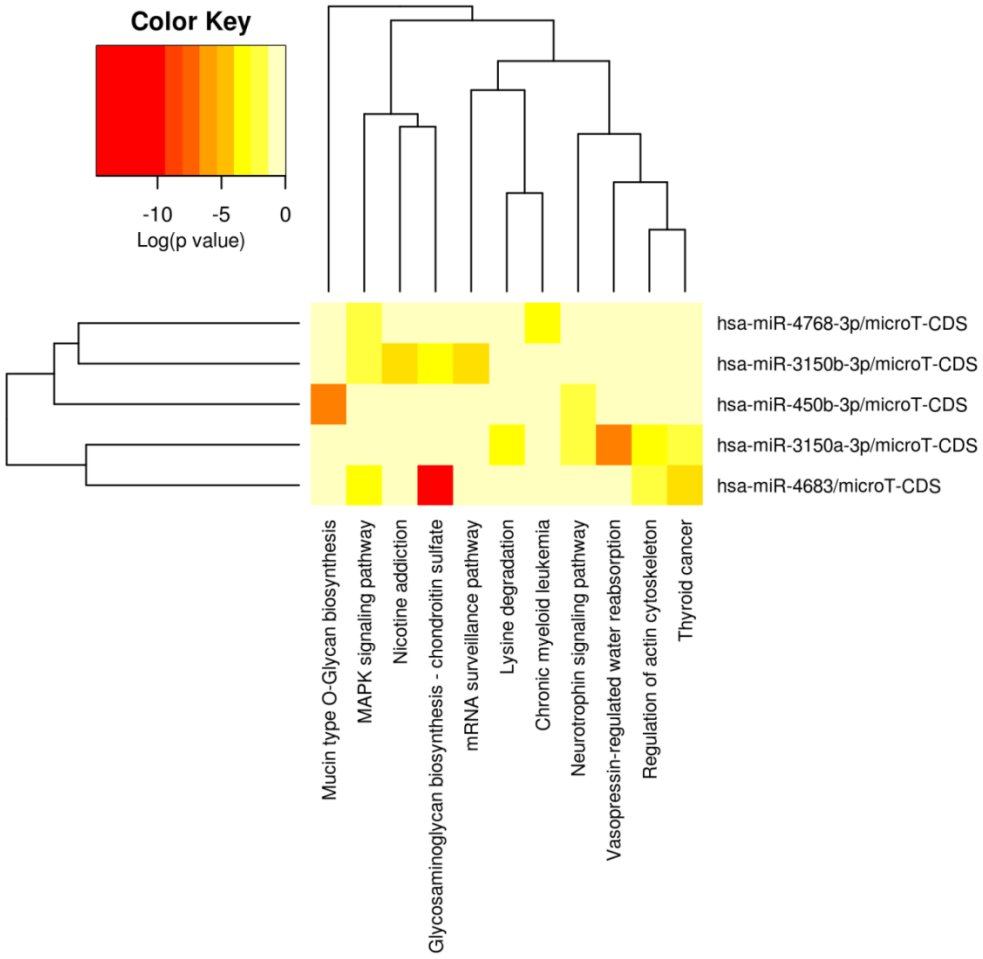


Figure 5.2: Heat map for microRNA-enriched common pathways using DIANA miRPath (Vlachos et al., 2012).

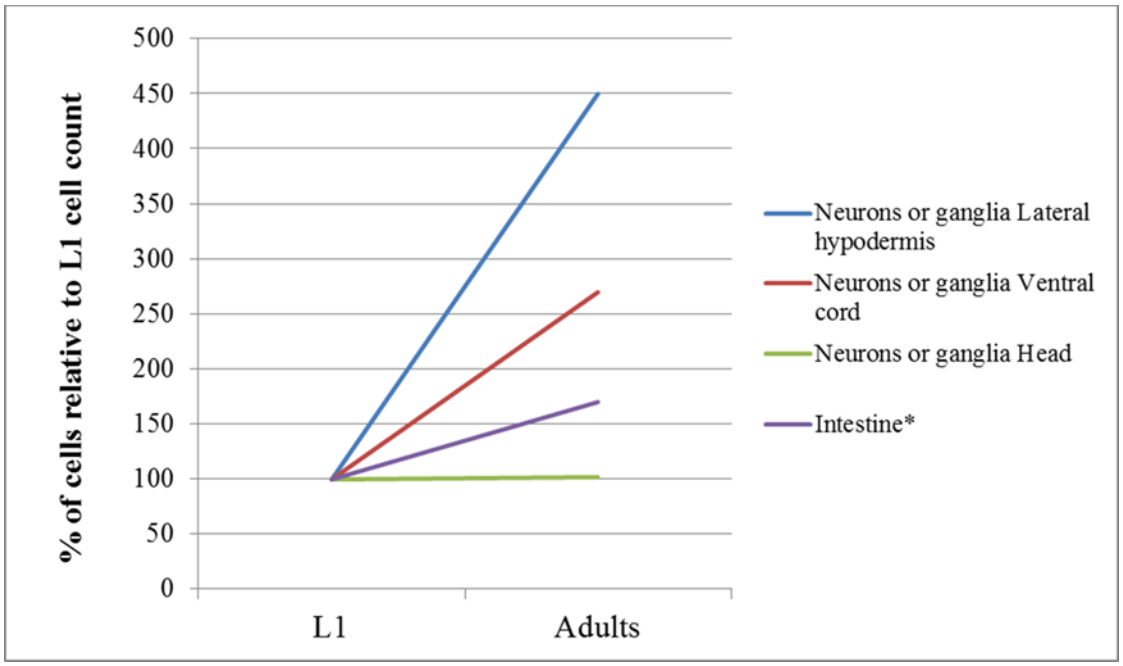


Figure 5.3: The percent increase in the number of cells from L1 to adult stage in neuronal and intestinal tissue. The number of cells was based on data from Sulston et al (J. E. Sulston, Schierenberg, White, & Thomson, 1983).