Published in final edited form as:

J Cell Physiol. 2014 January ; 229(1): 79–89. doi:10.1002/jcp.24419.

Chronic Nicotine Exposure Systemically Alters MicroRNA Expression Profiles during Post-embryonic Stages in *Caenorhabditis elegans*

Faten A Taki, Xiaoping Pan, and Baohong Zhang*

Department of Biology, East Carolina University, Greenville, NC 27858

Faten A Taki: TAKIF11@students.ecu.edu; Xiaoping Pan: PANX@ecu.edu

Abstract

Tobacco smoking is associated with many diseases. Addiction is of the most notorious tobacco-related syndrome and is majorly attributed to nicotine. In this study, we employed *C. elegans* as a biological model to systemically investigate the effect of chronic nicotine exposure on microRNA (miRNA) expression profile and their regulated biochemical pathways. Nicotine treatment (20 μ M and 20mM) was limited to the post-embryonic stage from L1–L4 (~31 hours) period after which worms were collected for genome-wide miRNA profiling. Our results show that nicotine significantly altered the expression patterns of 40 miRNAs. The effect was proportional to the nicotine dose and was expected to have an additive, more robust response. Based on pathway enrichment analyses coupled with nicotine-induced miRNA patterns, we inferred that miRNAs as a system mediates “regulatory hormones”, manifested in biphasic behavioral and physiological phenotypes. We proposed a model where nicotine addiction is mediated by miRNAs’ regulation of *fos-1* and is maintained by epigenetic factors. Thus, our study offers new insights for a better understanding of the sensitivity of early developmental stages to nicotine.

Keywords

nicotine; miRNA; *C. elegans*; dose-dependent; redundancy; addiction; regulatory hormones; biphasic response; post-embryonic exposure

Introduction

With reference to the 2010 Surgeon’s report, tobacco smoking damages every organ in the body and is eventually associated with disease and death. Consequently, 1000 persons are reported to die every day due to tobacco smoke-related health complications (CDC, 2010). Tobacco is a mixture of more than 4000 chemicals, most of which are carcinogenic. One of the toxic chemicals found in tobacco is nicotine that has been highly ranked among the addictive drugs of abuse. Interestingly, NIDA and others reported that children and adolescents are much more sensitive to nicotine dependence than adults (Kandel and Chen, 2000; Slotkin, 2002). With this in mind, the greater extent of exposure (Kim et al., 2009) and sensitivity in children stresses on the importance of evaluating the degree of effects on multiple levels.

Nicotine is known to be psychoactive and affects a variety of pathways including the mesolimbic reward pathways (Wada et al., 1989) in a non-monotonic, dose-dependent

*Corresponding Author: Dr. Baohong Zhang, Department of Biology, East Carolina University, Greenville, NC 27858, Phone: 252-328-2021, Fax: 252-328-4178, zhangb@ecu.edu.

manner. A biphasic response has been reported where lower doses caused stimulation, while higher doses were associated with a depressant-like effect on the nervous system (Benowitz, 1988).

Taken collectively, tobacco is a serious poison as it entraps users in a vicious cycle mediated by nicotine and eventually made worse by a variety of toxicants. As mentioned previously, smokers are more prone to damage in different organs and various diseases. Despite the in depth knowledge gained over the years regarding the effects on the pathological and behavioral levels, molecular investigations such as microRNA (miRNA) profiling remain in their juvenile stages. Investigating complex diseases in humans is not trivial and can be hindered by several factors (e.g. ethical concerns, genetic complexity). Therefore, public health demands have lead researchers to base their preliminary studies on simpler organisms with major conserved signaling pathways. For a more holistic understanding of nicotine's mechanism of action, we employed *C. elegans* as a biological model to study the effects of nicotine on the genome-wide miRNA expression patterns. We then tried to investigate the correlation of these altered miRNA profiles with their predicted regulatory biochemical pathways.

The genome encodes all physiological functions, but its expression is tightly regulated by a network of factors. Extensive 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. Interestingly, such environmentally-induced changes are mainly mediated by diverse epigenetic processes which in most cases result in heritable changes that do not involve changes in the DNA (Bird, 2007; Goldberg et al., 2007). Recently, epigenetics has been considered to be the link between the environment and the genome which might finally be reflected in emergent cellular processes. We are mainly interested in one of the epigenetic regulators, known as miRNAs (Zhang and Ho, 2011).

miRNAs are an extensive class of newly discovered small regulatory RNAs. In 1993, lin-4 was thought to be involved in lin-14 regulation via an RNA-RNA antisense interaction in *C. elegans* (Lee et al., 1993). Afterwards, miRNAs were ubiquitously discovered in almost all eukaryotic organisms (He and Hannon, 2004). Over 200 and 1000 miRNAs have been sequenced in *C. elegans* and humans, respectively. Initially reported to control the developmental timing in *C. elegans* (Ambros, 1989), their roles extended to diverse physiological and pathophysiological processes (Ambros, 2003; Aukerman and Sakai, 2003; Chen et al., 2004; Kim, 2005; McManus, 2003). Due to their conserved and pleiotropic roles in gene regulation, miRNAs are biomarkers of an innate response to environmental fluctuations. Thus, it is logical to study the effect of nicotine on the global miRNA profiles. Approaches like the latter provide further understanding on the pathologically physiological pathways of popular drugs of abuse like nicotine. To our knowledge, no previous study has been done on the impact of nicotine on the genome-wide miRNA expression in *C. elegans*. Our study offers new insights related to nicotine's dramatic effect when exposed during post-embryonic stages. Our aim was to identify possible miRNA patterns that are linked to nicotine-induced behavioral (e.g. addiction) and protein disorders (e.g. receptor desensitization).

Material and Methods

Chemicals and Strains

Purified nicotine was purchased from Acros Organics (NJ, USA). 1 M and 0.001 M stocks were prepared by diluting nicotine in phosphate buffer. Nicotine solution was introduced to molten NGM agar (Stiernagle, 2006) before being poured onto plates. NaCl, peptone, agar and water mixture were first autoclaved and kept at 70 °C covered under the hood. Equal

amounts were transferred to individual small autoclaved flasks cooled and kept at 55 °C. After adding cholesterol, CaCl₂, MgSO₄ and KH₂PO₄, nicotine solution was added to the corresponding final concentrations 20μM and 20mM in the medium. The dosages were selected based on our preliminary study as well previous reports (Feng et al., 2006b; Matta et al., 2007b; Sobkowiak et al., 2011; Waggoner et al., 2000b), in which nicotine treatment had a biphasic response.

C. elegans hermaphrodite N2 Bristol wild type was used. Maintenance and worm transfer were done after NGM plates were seeded with OP50 and then left to dry for ~ 10–15 minutes, and then kept at 20°C. *E. coli* stocks were stored as an LB pellet at –20 °C.

Egg synchronization was done via bleaching according to a standard method with slight modification (Sulston and Hodgkin, 1988). Briefly, adult gravid worms were washed off the plate with M9 buffer into a 15 ml falcon tube (for a medium sized pellet). Then the falcon tube was centrifuged at 2000 rpm for 2 minutes. The supernatant was then removed leaving the pellet. The wash was then repeated with 5 ml M9 followed by centrifugation and supernatant removal. Then, 5 ml of synchronization solution was added. The tube was shaken for 4 minutes until the adult worms burst leaving the eggs dispersed in solution (a maximum of 4–5 minutes in bleach solution). The tubes were then spun at 2000 rpm for 2 minutes. The supernatant was removed and three to four 5-ml M9 washes followed leaving the last wash without centrifugation. The tubes with the suspended eggs were placed on a shaker in the 20°C incubator for 14–18 hours maximum (to avoid starvation). After hatching, all progeny were stuck at L1. The latter were seeded plated onto treatment plates accordingly supplied with fresh OP50. Exposure lasted around 31 hours until end of L3–beginning of L4.

miRNA expression profile

Total RNA extraction was performed for all treatment groups according to protocol using mirVana™ miRNA Isolation Kit. Briefly, the sample was denatured using a lysis buffer. RNA was then separated from DNA and other 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 reversely transcribe RNA to cDNA for all 231 miRNAs. A total of 200ng of RNA was used for each RT reaction. The reactions were then run using thermal cycler for 16°C for 30 min followed by 42°C for 30 min, 85°C for 5 min and finally held at 4°C. The samples 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-plate using the ViiA™ 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, 1μL diluted cDNA, and 1μL primers. A minimum of 3 biological replicates were used. The reaction was run for 10 min at 95°C for enzyme activation followed by denaturation for 15 sec at 95°C and an annealing/extension step for 60 sec at 60°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 delta Ct (ΔCt) values were calculated as $Ct_{(miRNA)} - Ct_{(avg\ miRNAs)}$. The ΔΔCt was calculated as the difference in the

ΔC_t values between control and treatment. Then the fold change was calculated as $2^{(\Delta\Delta C_t)}$. Statistical analysis was based on t-test for independent samples via SPSS(20). The results were further narrowed based on two criteria. Only the genes whose expression changed with a $p < 0.05$ and a fold change ≥ 0.5 , when compared to control, were considered as differentially expressed and were subjected to further analysis.

Fold change values ($2^{(\Delta\Delta C_t)} - 1$) were used to construct heat maps coupled with non-supervised hierarchical clustering using Euclidean distance and single linkage analysis and included all genes and samples. The latter approach was done for both total miRNAs as well as miRNAs that underwent statistically significant expression alterations using MeV (MultiExperiment Viewer) (AI et al., 2006; Schmittgen et al., 2008).

Target prediction and pathway analysis

miRNAs that showed fold changes higher than ± 1 were used to perform target prediction using mirSOM software (Heikkinen et al., 2011). To prepare the input for analysis, duplicates were removed and thus only unique values of targets with 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 (DW et al., 2009a; DW et al., 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 et al., 2009) that provided DAG (directed acyclic graph) showing relationships among enriched processes. miRNA-target networks were constructed using cytoscape (Smoot et al., 2011).

Results

Genome-wide miRNA expression profiling

We studied the effect of nicotine on the expression levels of 231 miRNAs in L4 *C. elegans* (N2). The average of the total miRNA expression remained constant in each of the control and treatment groups (Figure 1A). Thus, it was used for normalization of the C_t values for the 231 miRNAs. Fold change values were calculated in comparison to control and were used to construct a heat map. After performing unsupervised hierarchical clustering, the high concentration treatment groups clustered together (Figure 1C). On the other hand, the low concentration treatment groups were ordered next to each other without being clustered. Complimentarily, the expression graphs of all miRNAs across treatment groups showed less variation in worms treated with the higher nicotine concentration in comparison to those treated with the lower one (Figure 1B).

To check for statistically significant alterations in the miRNA patterns, only miRNAs whose expression changed by more than 50% with ($p < 0.05$) were considered. A total of 40 miRNAs (17.3%) were significantly and consistently affected by nicotine treatment (Figure 1B). Then, the same unsupervised hierarchical clustering was performed coupled with leaf optimization for these 40 miRNAs (Figure 1D). Based on the new cluster, we noticed that the upper-limit distance for sample clustering decreased by about 31% due to the decrease in inter-sample variations. Similarly, groups exposed to the high nicotine concentration clustered together, while those exposed to the lower concentration still showed more variation and were therefore only closely ordered. In addition, taking the 1.39 distance as a cutoff, the miRNAs were binned into two major clusters. One cluster included 8 miRNAs (mir-1820, mir-358*, mir-55, mir-259, mir-235, mir-58, mir-1821, and lin-4). The remaining

miRNAs belonged to the second bin except for mir-2220 that clustered separately. Opposite patterns were characteristic of the two clusters. miRNAs belonging to the larger cluster were mostly upregulated (red color) in worms exposed to the higher nicotine concentration (20mM), while they were variable in the lower nicotine treatment groups. Additionally, miRNAs belonging to the smaller cluster were more downregulated in response to the higher nicotine treatment than they were in response to the lower nicotine concentration.

Quantitative assessment of differentially regulated miRNAs

Three miRNAs were altered in response to the lower nicotine concentration (20 μ M). Of them, miR-80 and miR-79 were upregulated by 1.0 ($p=0.045$) and 0.9 ($p=0.022$) folds, respectively. Conversely, miR-230* was inhibited by 0.5 folds ($p=0.019$). On the other hand, the expression of 38 miRNAs changed with statistical significance in worms treated with high nicotine concentration (20mM). The fold changes and p-values are shown in Table 1. About 78% of the altered miRNAs were upregulated with fold changes ranging from 0.5 to 3.4 folds. The most upregulated miRNAs were miR-2220 with 3.4 fold change ($p=0.034$) followed by mir-90 ($p=0.045$) and mir-2210 ($p=0.03$) with >1.5 folds change. Other miRNAs, such as mir-47* ($p=0.027$), mir-2216* ($p=0.034$), mir-49 ($p=0.019$), mir-38 ($p=0.004$), mir-255 ($p=0.003$), mir-1829b ($p=0.037$), mir-785 ($p=0.003$), and mir-241 (0.017), were up-regulated by at least 100%. The remaining 22% miRNAs that were affected by the 20mM nicotine treatment were down-regulated by at least 50%. The most down-regulated miRNAs were mir-58 ($p=0.014$) followed by mir-1821 ($p=0.03$) and lin-4 ($p=0.002$) (Table 1). In addition, miR-80 was the only miRNA upregulated in both low and high concentration nicotine treatments (Figure 2).

Functional analysis of differentially expressed miRNAs through target prediction and biochemical pathways analysis

Systematic miRNA→gene prediction→pathways—To investigate the potential function of these differentially expressed miRNAs in response to nicotine, we performed miRNA target prediction coupled with enrichment analyses based on two online software: DAVID and GOrilla. Thirteen miRNAs, with ± 1 fold changes, were used for target prediction. The number of gene targets varied among those miRNAs from tens to hundreds. In decreasing order, mir-47* was predicted to target 549 genes followed by mir-785 (521), mir-80 (501), mir-255 (265), mir-241 (224), mir-90 (167), mir-2220 (148), mir-2210 (140), mir-49 (127), mir-1829b (89), mir-38 (72), mir-242 (55), and mir-2216* (19) (Figure 4B). A unique list of 2395 genes was used as input for DAVID analysis. After functional annotation clustering, a ranked list of 321 genes was prepared based on the decreasing order of clusters with enrichment values 2.

DAG computed by GOrilla showed the enrichment of 5 major hubs (Figure 3). The “biological regulation” ($1.6, p<10^{-5}$), was generally divided into a molecular level summarized by metabolic and biosynthetic processes (e.g. RNA) and gene regulation (e.g. transcription). The second sub-level covered cellular and behavioral phenotypes such as neurogenesis and locomotion and location, respectively. Another major hub involved “response to stimulus” ($2.3, p<10^{-5}$) which was linked to neuro-related pathways through taxis. The highest enrichment was reported for immunity ($56.8, p<10^{-5}$) and was mainly reflected by response to other organisms (i.e. fungus). Also, one of the upstream nodes was “cellular process” ($1.11, p<10^{-5}$) and it branched to include growth, development, projection and organization, and recognition (e.g. axon guidance). The highest statistical significance was observed for “metabolic process” ($1.17, p<10^{-7}$) which comprised protein and phosphate-related modifications, single organism processes, and primary metabolism.

Enriched processes→genes→miRNAs—A bottom-top approach was then used to check the involvements of each of the 13 miRNAs in the enriched processes. Hence, we extracted the genes involved in 94 pathways outputted in GOrilla with p-values $< 10^{-3}$ (Figures 3 and 4). Then, we overlapped them with the predicted targets for each of the 13 miRNAs. miR-47* was the only miRNA predicted to target genes belonging to all of the 94 pathways. In decreasing order, mir-80, mir-255 and mir-241 covered 93%, 85%, and 82% of the processes and had a similar involvement pattern that did not include some cellular processes (e.g. recognition, migration, development, growth). A slightly different pattern was observed for mir-785 and mir-2220 which covered 75% and 62% of the pathways, respectively, but were not involved in immunity and development. The situation was the same for mir-38 (62%) and mir-90 (57%), but both were also less involved in response to stimulus. Four of the remaining miRNAs [mir-49 (37%), mir-2210 (32%), mir-1821b (18%), and mir-242 9%)] were not predicted to target genes involved in the nucleotide metabolism and biosynthesis or gene regulation. Instead, the target genes were more concerned with protein metabolism and modifications as well as response to stimulus. Finally, mir-2216* did not show any match with any of the functions.

Commonly targeted genes

Figure 5A shows the gene network constructed for the 13 miRNAs with high expression change and their targets. To simplify this network, a new gene network was obtained after only considering 13 commonly targeted genes (Figure 5B). Based on the originally compiled unique target list, only F40F11.2 (0.04%) was predicted to be targeted by 5 miRNAs. 0.5% of the genes were commonly targeted by 4 miRNAs, while 2% and 14% were common targets for only 3 and 2 miRNAs, respectively. Only the genes targeted by at least 4 miRNAs were used in network construction. From the most to least pleiotropic, mir-47* was predicted to target all 13 genes. mir-785 targeted all except for B0336.3. All of the 8 genes (B0336.3, C48A7.2, ceh-44, fos-1, let-75, ptc-1, sem-4, and tag-97) targeted by mir-241 were also targeted by mir-255. mir-255 also targeted F10D2.10. Additionally, ain-2, F40F11.2, sax-3, and tsp-14 were potentially targeted by mir-2210, whereas 1829b only potentially targeted 3 of them but not F40F11.2. Finally, mir-80 matched with only one gene (B0336.3), while mir-49 and mir-242 targeted F40F11.2 and F10D2.10, respectively. Together, they comprised an intricate network as shown in Figure 5B. Complementarily, we ran functional annotation via DAVID which summarized all functions associated with the genes of interest. The 13 genes were classified into four major ontologies: development and growth, reproduction, and transcriptional regulation (Table 2).

Five major enriched functional hubs

Based on GOrilla analysis, biological regulation, response to stimulus, immune processes, cellular and metabolic processes were the five major hubs that involved in *C. elegans* responded to nicotine exposure. After overlapping miRNA-target genes with the corresponding functional hub, we constructed a network that depicted the relationship between most of the highly regulated miRNAs with enriched nicotine-induced biological pathways. The network revealed a “nicotine-butterfly effect”, where most miRNAs were involved in the metabolic processes, while more specific miRNAs were involved in regulation of immune response (Figure 5C).

Discussion

Nicotine is a potent stimulant and a cholinergic agonist. There is no uniform standard molecular phenotype associated with nicotine. Its action is therefore not only context-dependent, but is also based on the dose and duration of its exposure (Schafer, 2002b). With smoking being so prevalent in countries in the Middle East (e.g. Lebanon), the chances of

persistent nicotine exposure among the younger groups remain high. According to a recent report by WHO, the highest ratio for male: female teenage smokers reached 66:54% in 2010 (WHO, 2012). Early developmental stages are more sensitive to any sort of stresses. When considering nicotine, the case is not different. It is reported that even a limited nicotine exposure during adolescence may lead to symptoms of dependence and that this sensitivity might be due to the neurochemical changes in the brain that is different from those of adults (CDC, 2010; Slotkin, 2002). Consequently, children exposed to nicotine are more prone to become smokers when they grow up, therefore initiating a vicious cycle of nicotine usage. We were interested in assessing the extent of the nicotine-induced alterations on simpler organisms such as *C. elegans* that enables extrapolations to higher organisms. In *C. elegans*, it has been documented that nicotine treatment is associated with hyper-contraction of body wall-muscles, stimulation of egg laying, increased pharyngeal pumping as well as a decrease in the efficiency of male spicule in mating (Matta et al., 2007a; Schafer, 2002b). However, no previous studies have investigated the impact of nicotine on the genome-wide miRNA profile. In this study, we limited nicotine exposure to the post-embryonic stage and investigated miRNA patterns as well as target predictions and networks occurring in the L4 stage in response to nicotine. Here we report that nicotine altered the expression of 17% of total miRNAs. Of them, 78% were dramatically up-regulated by 50–340% in response to high nicotine concentrations while the remaining miRNAs were down-regulated by less than 100% in both treatment groups.

Comparison between nicotine-induced behavioral versus miRNA responses

From the behavioral perspective, it has been reported that different nicotine concentrations and exposure durations correlated with a “biphasic” response in the treated organisms. The mean speed increased in worms exposed to lower nicotine concentrations (10–100 μ M); and then, the mean speed decreased when exposed to higher nicotine concentrations (10–30mM) (Sobkowiak et al., 2011). Nicotine psychopharmacologic effects can stimulate or depress a variety of processes (e.g. central and peripheral nervous, cardiovascular, endocrine systems) (USDHHS, 1988). All together were summarized as dose-dependent psychoactive effects (Shadel et al., 2000) ranging from skeletal muscle relaxation, increases in brain serotonin and pituitary hormone, etc. (USDHHS, 1988) to symptoms like tremor, nausea, and weakness (Benowitz, 1988). How does that overlap with molecular level alterations? Unlike the behavior, increasing nicotine doses did not reverse the miRNA expression patterns. However, the degree of fold change (1 versus 3.4 folds) as well as the number of altered miRNAs (1.3% versus 16.4%) increased with increasing nicotine concentrations. Such a dose-dependent response was also manifested in the heat map after hierarchical clustering by distinct color gradients for mir-2220 and the two other multi-miRNA clusters (Figure 1C,D). Thus, the molecular miRNA response was proportional and can generally be estimated as hyperbolic as a function of nicotine dose.

Understanding the molecular basis of nicotine-induced behavior

The relation between molecular and behavioral levels can be described as “regulatory hormesis”. In normal conditions, homeostasis prevails in an organism. After lower exposure of a stressor, it responds with moderation. Such an intermediate level of regulation (e.g. regulatory miRNAs) can be associated with a stimulatory or a beneficial phenotype. However, as the stressor increases, the regulative response is inflated and could become aberrant and depressive. An established example is the response to vaccination (e.g. positive reinforcing immunity) versus the response to primary exposure to high toxicant levels (e.g. overwhelmed immunity and shock) (Figure 6A).

Figures 4 and 5 revealed similar as well as distinct functional patterns associated with each of the most highly altered miRNAs. mir-2216* was the least involved while mir-47* was the

most pleiotropic miRNA. The remaining miRNAs were involved in one or more of the enriched pathways. Additionally, mir-80 was the only miRNA whose expression was differentially altered in response to the low nicotine dose. However, 12 other miRNAs were dramatically affected in response to the high nicotine concentration. All of mir-90, mir-38, mir-2220, mir-785, mir-241, mir-255, mir-80, and mir-47* co-regulate common processes (e.g. transcription). Clearly, the additive effect of 8 miRNAs would be associated with a more robust phenotype. For example, nicotine alters the expression levels of acetylcholine receptors. A small increase or decrease in the cell surface receptors might be associated with increased receptivity or saturation and therefore a resulting excitation of the downstream pathways such as muscle contraction. Conversely, a dramatic up-regulation or depression in the transcription of the receptors might lead to a permanent transduction or an inhibition of the signal. Such is consistent with previously reported studies where nicotine increased muscle contraction and egg laying and hindered male reproduction behavior and finally lead to muscle paralysis at high concentrations (Feng et al., 2006a; Sobkowiak et al., 2011; Waggoner et al., 2000a).

Interestingly, mir-80 belongs to the mir-58 family that includes mir-58, mir-80, mir-81, mir-82, and mir-1834 (Alvarez-Saavedra and Horvitz, 2010). The deletion of mir-80 was associated with healthy ageing, decrease in body and brood size (Vora, 2011). Therefore, one can anticipate a generally reversed phenotype in L4 worms exposed to nicotine during their larval stages. Nicotine can therefore have a negative effect on lifespan, while it alters the body size and reproduction and such can be partially mediated by nicotine-induced up-regulation of mir-80.

Nicotine induces addiction

A more holistic approach takes into consideration of all miRNAs as a system. A slight trigger can be inductive to certain pathways, while strong stimuli can render the system hyperactive and eventually problematic. However, induction is not necessarily positive as it may initiate or promote the activation of pathological pathways (e.g. drug dependence). Thus, it is intriguing to understand nicotine-linked phenotypes, particularly at the lower doses. Indeed, nicotine did cause an increase in locomotion and egg laying in *C. elegans* at 20 μ M (data is not shown). However, nicotine is also very addictive. Addiction is defined as a maladaptive form of neuroplasticity. The latter refers to the ability to adapt and respond to fluctuations in the environment. It promotes seeking and recognizing effectors important for survival while avoiding dangerous signals. However, long periods of stress (e.g. drugs of abuse) cause structural (e.g. alterations in nAChRs) (Fabian and Sonenberg, 2012) as well as molecular changes (Ender and Meister, 2010). Gene expression is regulated by a variety of players such as transcription factors. The Fos family of transcription factors is important for the induction and maintenance of long-term plasticity. It is induced and stabilized in response to wide range of acute stimuli, while it is desensitized after chronic treatment (e.g. drugs of abuse) (Ender and Meister, 2010). Histone modifications have been reported to have a role in promoting the transient versus permanent transcriptional states for fos-1 (Maze and Nestler, 2011). Moreover, fos-1 has a role in dendrite development and remodeling. Such was restricted to only a small time window during development and was not recapitulated when introduced during adulthood (Vonhoff et al., 2013). Interestingly, fos-1 was one of the genes predicted to commonly targeted by 4 of the affected miRNAs and might thus mediate an “addiction-like” behavior in *C. elegans* larvae. As a conclusion, we hypothesized a model that might explain nicotine addiction phenotype as shown in Figure 6B. Chronic nicotine treatment limited to the post-embryonic stages alters miRNA expression levels (e.g. mir-47*, mir-241, mir-255, and mir-785) which regulate fos-1 expression. The downstream effects would include remodeling of dendrite branching and disruption in axon guidance and other neurotransmitters and receptors. Overall, the effect of

miRNAs on *fos-1*, complemented by epigenetic modifications, may mediate nicotine addiction initiated during post-embryonic stages.

On the other hand, the highest shift in nicotine-induced fold changes was observed for *mir-2220*. However, *mir-2220* was not predicted to regulate any of the 13 commonly targeted genes. Perhaps, when miRNAs act redundantly, the additive increase in their expression levels fulfills the finetuning of their targets in response to the environment. On the contrary, when a single miRNA targets more specific genes, a more dramatic shift in its expression is observed to accommodate and respond to the new condition.

Conserved mechanism of action from *C. elegans* to humans

Statistically significant conservation was reported for 76 *C. elegans* miRNAs with 96 human miRNAs based on the 5' end sequence homology. Also, the overall length of 22 of these worm miRNAs were more than 70% homologous to 46 human miRNAs (Ibanez-Ventoso et al., 2008). In our study, 5 of the miRNAs (*mir-49*, *mir-80*, *mir-90*, *mir-241*, and *mir-785*) whose expression was strongly altered in response to nicotine have 5' end homology with 20 human miRNAs (Ibanez-Ventoso et al., 2008). Ibanez-Ventoso and colleagues showed that *mir-80* had only one human homologue *hsa-miR-450b-3p* (Ibanez-Ventoso et al., 2008). On the other hand, Fos protein family is an established effector of addiction in higher organisms (Koob and Volkow, 2010). This protein family has been implicated in nicotine withdrawal in human cell lines (Ichino et al., 1999). Taken together, the above summary of previous studies further support the conservation of major signaling pathways across species. Hence, we anticipate that nicotine's mechanism of action is analogous across species. It would be interesting to examine if similar patterns and effectors like *cel-mir-80/has-miR-450b-3p* and Fos-protein family have a role in nicotine-mediated addiction in humans.

Both *C. elegans* and our human share the similar mechanism response to nicotine exposure. Approximately 60%–80% of *C. elegans*' genes are conserved with humans (Vella, 2005). More importantly, the primary targets of nicotine are the nicotinic acetylcholine receptors (nAChRs), ligand-gated ion channels that are highly conserved across species (Duerr et al., 2008). The *C. elegans* genome encodes 28 nAChR genes and over one-third of the total 302 neurons are involved in cholinergic transmission in adult hermaphrodite (Feng et al., 2006b; Schafer, 2002a). *C. elegans* also exhibits similar behavioral patterns as humans when exposed to nicotine, including withdrawal, tolerance, and sensitization (Feng et al., 2006b; Schafer, 2002a). Thus, *C. elegans* has long been used as an ideal model system to study nicotine addiction.

We limited our analysis based on criteria described above in an attempt to buffer possible signal from noise. Our data showed that nicotine altered the profiles of several miRNAs predicted to have pleiotropic roles. Therefore, its impact would be expected to be extensive and involves major pathways. miRNAs are known to co-target many genes. Researchers interpreted such a phenomenon as a mean to fine tune gene expression and provide robustness against environmental perturbations. Rather than completely switching gene expression, miRNAs' impact is cumulative. Together they fine tune and stabilize regulatory networks to establish relatively normal physiologies in response to fluctuating environments (Li et al., 2009).

Acknowledgments

The project described is partially supported by Grant Number R03DA032515 from the National Institute on Drug Abuse. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute on Drug Abuse or the National Institutes of Health.

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Figure 1.

(A) Centroid graph showing the stability of the total mean fold change of the 231 miRNAs. (B) Expression graphs for 231 miRNAs (top) and 40 miRNAs that showed differential expression in response to nicotine (bottom). Each line represents one the expression of one miRNA across the treatment groups. (C) Heat map showing expression profiles of 231 miRNAs. (D) Heat map of 40 miRNAs considered to be differentially regulated. Hierarchical clustering was performed with Euclidean distance based on single linkage for each of the miRNAs among all the treatment groups. Note: from left to right, three biological replicates of each: 20 μ M (L), and 20mM (H) treatment groups. Each cell represents a MFC compared to control (Mean Fold Change: $2^{(\Delta\Delta CT)} - 1$). In the figures, color red, green, and black represents up-regulation, down-regulation and no change, respectively. Heat maps and clustering were done using Mev software.

Figure 2.

(A) Nicotine treatment was associated with altered expression of 40 miRNAs in L4 *C. elegans* (N2). miRNA expression is represented as fold change $2^{\Delta\Delta Ct}$. ($\Delta\Delta Ct = \Delta Ct_{\text{control}} - \Delta Ct_{\text{treatment}}$). miRNAs whose expression changes with a $p < 0.05$ and fold change ≥ 0.5 and ≤ -0.5 , respectively. (*) and (β) denote $p < 0.05$ for the high and low treatment groups, respectively in comparison to control. B) Venn diagram showing the number of miRNAs whose expression was altered in response to low, high, or both nicotine concentrations.

Figure 3. Directed acyclic graph (DAG) performed by GOrilla. It shows the relationships among the enriched pathways targeted by miRNAs altered in response to nicotine treatment. Colors represent P-values. From white to dark orange, P-values range from $>10^{-3}$ to $<10^{-9}$.

Figure 4.

Nicotine-induced miRNAs differentially regulate five major biological pathways. (A) summary of pathways enriched by GOrilla based on the predicted target genes for the 13 most highly altered miRNAs (MCF>1). The data labels at the edge of each bar represent the p-values calculated for each enrichment value. (B) Functional distribution of the 13 most highly altered miRNAs in response to nicotine. Each miRNA was associated with none, one or more, or all of the enriched pathways. Next to each miRNA name () is the number of targets predicted for each miRNA by mirSOM.

Figure 5.

(A) A miRNA-target network showing the pleiotropic and redundant nature of all of the highly altered miRNAs in response to nicotine treatment in L4 *C. elegans* (N2). The network included 13 miRNAs and 2877 predicted targets. (B) Only genes commonly targeted by at least 4 of the differentially altered miRNAs were used. Thus 13 miRNAs and 13 gene targets were used to construct this network. (C) Nicotine-butterfly effect shows possible matches among each of the highly altered miRNAs with the five enriched functional hubs (GORilla). Networks were constructed by Cytoscape. Each red dot represents a match between one miRNA and one hub.

Figure 6.

A miRNA system mediates “regulatory hormesis” and addiction. (A) As nicotine concentration increases, the number of affected miRNAs increases. The latter is associated with a biphasic behavioral and physiological phenotype. (B) A proposed model that explains nicotine addiction as a function of miRNAs’s regulation of fos-1 and maintenance of effect by epigenetic factors.

Table 1

A summary of differential miRNA-fold change ($\pm$ SE) in response to low (20 μ M) and high (20mM) nicotine treatments in L4 *C. elegans* (N2).

	Low		High	
	P value	Fold change $\pm$ SE	P value	Fold change $\pm$ SE
*Lin4	0.761	0.12 $\pm$ 0.34	0.002	-0.56 $\pm$ 0.08
*miR1018	0.375	0.33 $\pm$ 0.29	0.013	0.54 $\pm$ 0.13
*miR1022	0.491	0.67 $\pm$ 0.80	0.040	0.58 $\pm$ 0.12
*miR1817	0.627	0.10 $\pm$ 0.18	0.038	0.70 $\pm$ 0.23
*miR1820	0.861	-0.07 $\pm$ 0.36	0.000	-0.49 $\pm$ 0.03
*miR1821	0.669	-0.15 $\pm$ 0.31	0.030	-0.58 $\pm$ 0.10
*miR1829b	0.839	-0.13 $\pm$ 0.57	0.037	1.10 $\pm$ 0.36
*miR1833	0.723	0.11 $\pm$ 0.26	0.018	0.82 $\pm$ 0.21
*miR2209a	0.796	-0.08 $\pm$ 0.26	0.000	0.47 $\pm$ 0.02
*miR2210	0.207	0.57 $\pm$ 0.31	0.030	1.55 $\pm$ 0.28
*miR2215	0.772	0.09 $\pm$ 0.28	0.035	0.79 $\pm$ 0.15
*miR2216St	0.235	1.05 $\pm$ 0.62	0.034	1.32 $\pm$ 0.25
*miR2218aSt	0.814	0.07 $\pm$ 0.28	0.024	0.74 $\pm$ 0.12
*miR2218bSt	0.405	0.52 $\pm$ 0.56	0.036	0.91 $\pm$ 0.29
*miR2220	0.465	0.52 $\pm$ 0.58	0.034	3.44 $\pm$ 0.65
β miR230St	0.019	-0.49 $\pm$ 0.07	0.600	0.27 $\pm$ 0.44
*miR235	0.935	0.03 $\pm$ 0.28	0.043	-0.52 $\pm$ 0.11
*miR241	0.753	0.16 $\pm$ 0.45	0.017	1.02 $\pm$ 0.14
*miR242	0.469	0.47 $\pm$ 0.59	0.035	0.99 $\pm$ 0.19
*miR243	0.371	-0.09 $\pm$ 0.08	0.025	0.64 $\pm$ 0.10
*miR255	0.981	-0.01 $\pm$ 0.59	0.003	1.19 $\pm$ 0.14
*miR258	0.351	-0.23 $\pm$ 0.19	0.021	0.47 $\pm$ 0.07
*miR259	0.492	-0.14 $\pm$ 0.18	0.002	-0.49 $\pm$ 0.07
*miR358St	0.316	-0.26 $\pm$ 0.23	0.023	-0.49 $\pm$ 0.08
*miR38	0.359	0.23 $\pm$ 0.22	0.004	1.20 $\pm$ 0.08
*miR47St	0.164	0.74 $\pm$ 0.34	0.027	1.34 $\pm$ 0.22
*miR49	0.984	-0.01 $\pm$ 0.45	0.019	1.30 $\pm$ 0.18
*miR54	0.712	0.07 $\pm$ 0.16	0.023	0.89 $\pm$ 0.14
*miR55	0.081	-0.36 $\pm$ 0.11	0.003	-0.52 $\pm$ 0.03
*miR58	0.623	0.19 $\pm$ 0.35	0.014	-0.67 $\pm$ 0.08
*miR76	0.885	-0.06 $\pm$ 0.36	0.029	0.59 $\pm$ 0.10
*miR785	0.220	0.47 $\pm$ 0.30	0.003	1.08 $\pm$ 0.16
*miR789	0.076	0.64 $\pm$ 0.19	0.014	0.49 $\pm$ 0.12
β miR79	0.022	0.86 $\pm$ 0.20	0.392	-0.11 $\pm$ 0.10
*miR794	0.617	0.19 $\pm$ 0.33	0.000	0.56 $\pm$ 0.02

	Low		High	
	P value	Fold change $\pm$ SE	P value	Fold change $\pm$ SE
*miR798	0.970	-0.01 ± 0.26	0.032	0.89 ± 0.16
*miR799	0.512	-0.15 ± 0.19	0.014	0.46 ± 0.05
β*miR80	0.045	1.01 ± 0.30	0.011	0.86 ± 0.09
*miR800	0.755	0.11 ± 0.31	0.016	0.60 ± 0.15
*miR90	0.251	0.33 ± 0.23	0.045	1.69 ± 0.37

(* and β) denote $p < 0.05$ in response to high and low nicotine doses, respectively.

Table 2

A list of proposed genes that are targeted by 4 miRNAs. Gene descriptions were summarized from DAVID and WormBase (Yook et al., 2012).

Gene symbol	Description	Function
ain-2	ain-1 paralogue	no obvious function in mass RNAi assays
B0336.3		body morphogenesis, regulation of growth, regulation of growth rate, positive regulation of growth rate, positive regulation of growth
C48A7.2		reproductive developmental process, ion transport, phosphate transport, anion transport, intracellular protein transport, sex differentiation, protein localization, embryonic development ending in birth or egg hatching, protein transport, inorganic anion transport, vesicle-mediated transport, cellular protein localization, negative regulation of vulval development, regulation of vulval development, hermaphrodite genitalia development, establishment of protein localization, intracellular transport, regulation of post-embryonic development, negative regulation of post-embryonic development, genitalia development, cellular macromolecule localization
ceh-44	Homeobox	transcription, regulation of transcription, DNA-dependent, intra-Golgi vesicle-mediated transport, vesicle-mediated transport, regulation of transcription, intracellular transport, Golgi vesicle transport, regulation of RNA metabolic process
F10D2.10		Unknown function, lipase putative
F40F11.2		embryonic development ending in birth or egg hatching, oxidation reduction
fos-1	FOS (B-Zip transcription factor) homolog	cell fate specification, morphogenesis of an epithelium, reproductive developmental process, regulation of transcription, DNA-dependent, regulation of transcription from RNA polymerase II promoter, sex differentiation, positive regulation of biosynthetic process, positive regulation of macromolecule biosynthetic process, positive regulation of gene expression, positive regulation of cellular biosynthetic process, regulation of gene-specific transcription, hermaphrodite genitalia development, positive regulation of gene-specific transcription, cell fate commitment, regulation of transcription, positive regulation of transcription, DNA-dependent, positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process, positive regulation of transcription, positive regulation of transcription from RNA polymerase II promoter, tissue morphogenesis, genitalia development, positive regulation of nitrogen compound metabolic process, regulation of RNA metabolic process, positive regulation of RNA metabolic process, regulation of syncytium formation by plasma membrane fusion, epithelium development
let-75	LEThal	nematode larval development, larval development, muscle system process, muscle contraction, post-embryonic development, embryonic development ending in birth or egg hatching, growth
ptc-1	PaTChed family	M phase of mitotic cell cycle, mitotic cell cycle, M phase, nuclear division, nematode larval development, larval development, cell cycle, mitosis, behavior, post-embryonic development, embryonic development ending in birth or egg hatching, molting cycle, protein-based cuticle, oviposition, molting cycle, collagen and cuticulin-based cuticle, reproductive behavior, cell cycle process, cell cycle phase, multicellular organism reproduction, reproductive behavior in a multicellular organism, growth, molting cycle, organelle fission, reproductive process in a multicellular organism, cell division
sax-3	Sensory AXon guidance	regulation of growth, regulation of growth rate, positive regulation of growth rate, positive regulation of growth
sem-4	SEx Muscle abnormal	behavior, oviposition, reproductive behavior, multicellular organism reproduction, reproductive behavior in a multicellular organism, regulation of growth, regulation of growth rate, positive regulation of growth rate, positive regulation of growth, reproductive process in a multicellular organism
tag-97	Temporarily Assigned Gene name	regulation of transcription, DNA-dependent, regulation of transcription, regulation of RNA metabolic process
tsp-14	TetraSPANin family	defense mechanisms