Published in final edited form as:

Reprod Toxicol. 2013 September ; 40: 69–75. doi:10.1016/j.reprotox.2013.05.007.

Impacts of chronic low-level nicotine exposure on *Caenorhabditis elegans* reproduction: Identification of novel gene targets

Michael A. Smith Jr.^a, Yanqiong Zhang^a, Joseph R. Polli^a, Hongmei Wu^a, Baohong Zhang^a, Peng Xiao^b, Mary A. Farwell^a, and Xiaoping Pan^{a,*}

^a Department of Biology, East Carolina University, Greenville, NC 27858, USA

^b Department of Mathematics, East Carolina University, Greenville, NC 27858, USA

Abstract

Effects and mechanisms of chronic exposure to low levels of nicotine is an area fundamentally important however less investigated. We employed the model organism *Caenorhabditis elegans* to investigate potential impacts of chronic (24 h) and low nicotine exposure (6.17–194.5 μ M) on stimulus-response, reproduction, and gene expressions. Nicotine significantly affects the organism's response to touch stimulus ($p = 0.031$), which follows a dose-dependent pattern. Chronic nicotine exposure promotes early egg-laying events and slightly increased egg productions during the first 72 h of adulthood. The expressions of 10 (*egl-10*, *egl-44*, *hlh-14*, *ric-3*, *unc-103*, *unc-50*, *unc-68*, *sod-1*, *oxi-1*, and *old-1*) out of 18 selected genes were affected significantly. Other tested genes were *cat-4*, *egl-19*, *egl-47*, *egl-5*, *lin-39*, *unc-43*, *pink-1*, and *age-1*. Changes in gene expression were more evident at low dosages than at relatively high levels. Genes implicated in reproduction, cholinergic signaling, and stress response were regulated by nicotine, suggesting widespread physiological impacts of nicotine.

Keywords

Nicotine; Chronic exposure; *C. elegans*; Reproduction; Gene expression; Stress-response

1. Introduction

Nicotine, a major component of tobacco products, is one of the most abused substances that lead to addiction. Nicotine targets nicotinic acetylcholine receptors (nAChRs), stimulates the mesolimbic dopamine system, and results in dependence [1,2]. Chronic exposure to nicotine in heavy smokers or via second hand smoking is a major component for developing addiction behaviors. It is known that long-term exposure to nicotine causes overexpression of nAChRs, especially the heteromeric receptors, contributing to the adaptive mechanism essential for drug-dependence and addiction [3]. Long-term smoking is also associated with various chronic disorders including several cancer types, cardiovascular diseases, and chronic obstructive pulmonary disease (COPD). These may be attributed to the cumulative oxidative stress, DNA damage, fatty acid buildup, and inflammatory status [4,5]. In

© 2013 Elsevier Inc. All rights reserved.

* Corresponding author at: N108 Howell Science Complex, Greenville, NC 27858, USA. Tel.: +1 252 328 5443 16; fax: +1 252 328 4718. Panx@ecu.edu (X. Pan)..

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

addition, studies have noted a positive relationship between increased mortality rate and prenatal nicotine exposure [6]. As a result, cigarette smoking is one of the most important causes of premature death and disability world-wide [7]. Thus, investigating mechanisms of nicotine effects and seeking novel biochemical targets has long been an interest of drug industry, in an effort to develop early intervention strategies.

The model organism *Caenorhabditis elegans* has been widely used to decipher mechanisms of action of various abused substances including nicotine. Compared with other animal model systems, *C. elegans* has many unique advantages, including a completely decoded genome, short life cycle, and large brood size. The *C. elegans* genome encodes 28 nAChR genes and over one-third of the total 302 neurons in an adult hermaphrodite are involved in cholinergic transmission, which is important for regulating various drug effects [8,9]. Besides the nAChR genes, approximately 60–80% of *C. elegans*' genes are conserved with humans [10]. In addition, the reproductive process of gametogenesis has been clearly demonstrated in *C. elegans*, make it an ideal model system to study reproductive effects of toxicants/drugs. All these advantages facilitate the study of the genetic basis of nicotine toxicity *in vivo*.

It is known that the nAChR agonist nicotine stimulates body wall muscle contraction and promote egg-laying behaviors in *C. elegans* [11]. Acute exposure to nicotine at high levels causes paralysis of the body wall muscle, while chronic exposure to high nicotine dosage results in alterations of egg-laying behaviors. *C. elegans* wild-type (wt) animals lay eggs in a temporal pattern: egg-laying tends to be clustered in short clustered episodes, which are separated by longer intervals during which eggs are not laid [12]. Chronic exposure to a relatively high concentration of nicotine (30 mM) results in time shortening of the egg-laying events and elongation of the intercluster time interval [13,14]. Feng et al. firstly defined nicotine-dependent behaviors following acute and chronic exposure (16 h) to low levels of nicotine (0.5–5 μ M) [9]. Another study revealed that nicotine exposure induces concentration-dependent (at a dose range of 0.001–30 mM) and time-dependent (from 0 min to 300 min) changes in locomotion behaviors [15]. However, effects of chronic nicotine exposure on egg-laying behaviors and potential impacts on reproduction remain largely unknown. The *C. elegans* egg-laying circuit consists of at least three types of cells: the vm2 vulval muscles, and the hermaphroditespecific HSN and VC motoneurons (reviewed by [16]). The muscle cells receive synaptic input from neurons through the action of various neurotransmitters (mainly acetylcholine, serotonin, and neuropeptides) on corresponding membrane receptors. Although researches have identified many genes that play important roles in egg-laying and reproductive aspects, few studies have worked on the nicotine-induced gene expression pattern, specifically on identifying key gene players responding to chronic low nicotine exposures.

In this study, we employed *C. elegans* as a model organism to investigate the effect of chronic low nicotine exposure on the response to stimulus, reproduction, and gene expression of eighteen selected genes implicated in egg-laying, locomotion, and stress-response.

2. Materials and methods

2.1. *C. elegans* culture and treatment

Wild-type *C. elegans* strain Bristol N2 were grown in Petri dishes on 6-cm nematode growth medium (NGM) and fed with *Escherichia coli* strain OP50 according to a standard protocol [17]. Worms at larval stage 3 (L3) from an age-synchronized culture were used in all experiments. Age-synchronized cultures were obtained according to a previous report [18]. Worms were then placed on an NGM plate to allow hatching with no food for an arresting

period to ensure all worms were closely synchronized. The worms were then transferred to OP50 seeded NGM agar plates, allowed developing into L3 stage (32–36 h after L1), and then collected for nicotine dosing.

L-Nicotine (98% pure) was obtained from Fisher Scientific. Different concentrations of nicotine dosing solution were made in K-medium (0.032 M KCl and 0.051 M NaCl): 1 ppm, 3.16 ppm, 10 ppm, and 31.6 ppm, corresponding to 6.17, 19.5, 61.7, and 194.5 μ M, respectively. K-medium was used as a control. L3 stage worms were exposed to each of five different dosages for 24 h with *E. coli* OP50 as food.

2.2. *C. elegans* recovery from dosing

After 24 h of exposure, worms were collected, rinsed, and transferred to 12 well tissue culture plates filled with OP50-seeded NGM agar. The worms were allowed to acclimate for 1 h and then observed for general morphology and movement using a dissecting microscope. If a worm was not moving it was touched with a platinum wire. Worms not responding to gentle platinum wire stimulation were counted as failure to recovery from dosing. Around 50 worms were placed in each well in triplicate for one biological replicate. Four biological replicates (four independent experiments) were performed for totaling ~600 worms per dose.

2.3. Effect of nicotine exposure on *C. elegans* 24-h egg-laying pattern

After 24 h of nicotine exposure, worms were collected, rinsed, and individually placed into 12-well OP50-seeded NGM plates for egg-laying. Eggs and hatched larvae were counted every 24 h for 72 h post-dosing. Three independent experiments were performed with three replicates per dosage each experiment, totaling nine replicates per dosed group for statistical purpose.

2.4. Effect of nicotine exposure on protein-coding gene expression in *C. elegans*

After 24 h of nicotine exposure, worms were collected and rinsed with M9 buffer 3–4 times to remove residual nicotine and bacteria. Worms were frozen in liquid nitrogen and then stored in at -80°C until RNA extraction.

Total RNA was isolated using the TRI Reagent® Reagent from Applied Biosystems according to the manufacturer's protocol. Total RNA was then quantified and assessed for quality using a Nanodrop ND-1000 (Nanodrop Technologies). Eighteen protein-coding genes (*cat-10*, *egl-10*, *egl-19*, *egl-44*, *elg-47*, *egl-5*, *hlh-14*, *lin-39*, *ric-3*, *unc-103*, *unc-43*, *unc-50*, *unc-68*, *sod-1*, *pink-1*, *oxi-1*, *age-1*, and *old-1*) that relate to worm reproductive traits and stress response were selected to test the impact of nicotine exposure on gene expression.

Applied Biosystems TaqMan microRNA Assays were employed to detect and quantify *C. elegans* protein-coding genes using quantitative real time PCR (qRT-PCR) according to the manufacturer's instructions. There were two steps in the TaqMan miRNA Assays: (a) reverse transcription of mRNA to a cDNA sequence using a poly(T) primer for protein-coding gene, and (b) quantitative real-time PCR. In short, single-stranded cDNA was generated from 500 ng of the total RNA from control, 1 ppm, 3.16 ppm, 10 ppm, and 31.6 ppm nicotine exposed *C. elegans* samples. Reverse transcription was carried out in a 15 μ L solution, which contained 500 ng of total RNA, 1 mM each of dNTPs, 1 μ L MultiScribe Reverse Transcriptase (50 U/ μ L), 1.5 μ L 10 $\times$ RT Buffer, 0.188 μ L RNase Inhibitor, and 3 μ L 5 $\times$ Taqman RT primer. The reverse transcription reaction was performed with an Eppendorf Mastercycler Personal PCR machine (Westbury, NY) with the following temperature program: initial 16 $^{\circ}\text{C}$ for 30 min followed by 42 $^{\circ}\text{C}$ for 30 min; then, the reaction was held for 5 min at 85 $^{\circ}\text{C}$; finally held at 4 $^{\circ}\text{C}$ until next analysis or stored at -20°C .

°C. For qRT-PCR analysis, each reaction was performed in a 20 μ L solution, which contained 2 μ L of RTPCR product (10-fold dilution from RT-PCR), 10 μ L of SYBR green PCR Master Mix, 2 μ L of forward and reverse primer and 6 μ L of DNase/RNase free water. The temperature program for qRT-PCRs was 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s.

All reactions had three technical replicates and each dose had three biological replicates. Supplemental Table 1 shows the primer information of the 18 tested genes. In qRT-PCR, Y45F10D, a conserved iron binding related protein was employed as an endogenous reference gene for normalizing qRT-PCR results [19]. Relative gene expression was analyzed using the $\Delta\Delta C_t$ method [20].

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.reprotox.2013.05.007>.

2.5. Statistical analysis

Statistical differences between the control and exposed worms were determined using standard statistical software (SPSS). Analysis of variance (ANOVA) was used for comparing means of different treatment groups. For time-series data obtained from egg-laying assays, repeated measures ANOVA using time as the factor were employed to test differences among treatment groups. If there was a significant difference among treatment groups at $p < 0.05$ level, least significant difference (LSD) multiple comparisons were conducted to compare means of each group.

3. Results

3.1. *C. elegans* recovery from dosing

The recovery rate of *C. elegans* was significantly affected by nicotine exposure ($p = 0.031$). After 24 h of exposure and a 1 h acclimation period, the percentage of *C. elegans* recovery decreased as the dose increased. The “No response to touch” rate was increased as nicotine concentration increased (Fig. 1). Nicotine concentrations equal to and larger than 3.16 ppm (19.5 μ M) significantly affect the recovery rate as compared to control. There was a dose-dependent increase in the percentage of animals that failed to respond to touch stimulus after dosing. From low to high concentrations, there were four distinct statistical groups defined including control (represented as a, ab, b, c and d on Fig. 1).

3.2. Effect of nicotine exposure on *C. elegans* 24-h egg-laying pattern

We did not observe eggs or hatched larvae in the dosing solution during nicotine exposure. During the first 24 h post-dosing, there was no significant difference in egg production among control and treatment groups. The average numbers of eggs laid (counted as eggs plus hatched larvae) during the first 24 h post-dosing for control and treatments were around 73 (Fig. 2). More eggs were laid during the second 24-h period (24–48 h post-dosing) (Fig. 2); the average numbers of eggs laid for control and treatment groups were around 112. The 10 ppm dosed group produced more eggs laid compared to the control; with a 10% increase ($p < 0.05$) during the second 24-h period. Other treatments did not significantly affect the egg production during this period. The number of eggs laid during the third 24-h period (48–72 h post-dosing) dropped, the average numbers of eggs laid for control and treatment groups were around 84 (Fig. 2). The highest treatment group (31.6 ppm) produced ~6% more eggs laid than control and the increase was significant ($p < 0.05$). Other treatments did not affect the egg production during this period. Fig. 3 shows the accumulated total of eggs laid over time. No significant difference observed among the two low nicotine treatment groups (1 ppm and 3.16 ppm) and control, however high concentrations (10 and 31.6 ppm)

of nicotine caused worms to produce significantly more eggs after 48 and 72 h post-dosing (Fig. 3). At 72 h post-dosing, the control group produced 261 eggs per worm while worms exposed to 1, 3.16, 10 and 31.6 ppm nicotine produced 265 ($p = 0.677$), 265 ($p = 0.645$), 276 ($p = 0.009$), and 277 ($p = 0.009$) eggs, respectively. About 4% more eggs were produced in the 10 ppm and 31.6 ppm groups during the 72 h period after nicotine exposure.

3.3. Effect of nicotine exposure on protein-coding gene in *C. elegans*

This study identified novel gene targets of nicotine by using a candidate gene approach; 18 candidate genes were selected for testing, including *cat-4*, *egl-10*, *egl-44*, *egl-47*, *egl-5*, *hlh-14*, *lin-39*, *ric-3*, *unc-103*, *unc-43*, *unc-50*, *unc-68*, *sod-1*, *pink-1*, *oxi-1*, *age-1*, and *old-1*. Table 1 shows the functional classification of tested genes. Chronic exposure to nicotine at the micromolar range induced aberrant gene expressions in wt *C. elegans* across all dosed concentrations. Fig. 4 shows the fold change in gene expression of the 18 tested genes, varying from 12.5-fold down-regulation (*old-1*) to 138.1-fold up-regulation (*hlh-14*). Statistical analysis indicated that there were 10 tested genes expressed aberrantly in response to at least one dosage of nicotine. These 10 genes are *egl-10*, *egl-44*, *hlh-14*, *ric-3*, *unc-103*, *unc-50*, *unc-68*, *sod-1*, *oxi-1*, and *old-1*. Among the 10 genes, 8 genes (*egl-10*, *egl-44*, *hlh-14*, *ric-3*, *unc-103*, *unc-50*, *unc-68*, and *oxi-1*) were significantly up-regulated, 2 genes (*sod-1* and *old-1*) were significantly down-regulated. Gene regulation is the most active at two low nicotine treatment groups; 3 (*egl-44*, *ric-3*, and *old-1*) and 7 tested genes (*egl-10*, *hlh-14*, *unc-103*, *unc-50*, *unc-68*, *sod-1*, and *oxi-1*) were differentially expressed at 1 ppm and 3.16 ppm treatment groups, respectively. In contrast, only 3 (*egl-44*, *ric-3*, and *old-1*) and 1 tested genes (*ric-3*) were significantly changed at 10 ppm and 31.6 ppm treatment groups, respectively. Changes in gene expressions were the most significant under 3.16 ppm nicotine exposure; six genes (*egl-10*, *hlh-14*, *unc-103*, *unc-50*, *unc-68*, and *oxi-1*) displayed at least 10-fold significant up-regulation.

4. Discussion

Acute nicotine exposure at high concentrations (20–30 mM) causes rapid paralysis of *C. elegans* body wall muscle [13]. Since nicotine exposure in humans also occurs at a low dosage range and last for a prolonged period of time, it is important to investigate effects of chronic exposure to low concentrations of nicotine. Human nicotine blood concentration reaches ~500 nM following the consumption of one cigarette [21]. In habitual smokers, the blood nicotine level ranges from ~30 to ~300 μ M [22]. In *C. elegans*, although nicotine-dependent behaviors were observed following chronic exposure (16 h) to low nicotine levels (500 nM–5 μ M)[9], the characterization of genetic changes following exposure has been less completed.

In this study we observed a dose-dependent loss-of-response to stimuli in *C. elegans* exposed to low concentrations (micromolar range) of nicotine for 24 h in liquid medium. Worms that failed to respond appeared rigid, straight or exhibited a boomerang-shaped body line, suggesting chronic nicotine exposure may cause paralysis even at micromolar concentrations. This may due to the increased cuticle permeability under chronic exposure and during molting between larval stages.

It is known that nicotine exposure affects egg-laying behaviors [13,14]. However, it is not clear that if nicotine affects eggs production. At 20 °C, *C. elegans* begins to lay eggs as they become young adults (~65 h after hatching) and the total egg-laying event lasts for about 2.5 days, ending ~128 h post-hatching [23]. As most eggs are produced during the first 72 h of adulthood, we used the first 3-day egg productions as a more sensitive reproduction effect for comparisons. We found that during the 24–48 h period after exposure, both control and nicotine-treated *C. elegans* produce significantly more eggs than the first and the third 24 h

period. This is consistent with the fact that the egg-laying maximal of wt *C. elegans* occurs ~31 h after the egg-laying begins [23]. Chronic exposure to 10 ppm nicotine seems to promote earlier egg-laying; as during the second 24 h period, 10 ppm group worms laid ~10% more eggs than others ($p < 0.05$). By 72 h post-dosing, worms treated with 10 ppm (61.7 μ M), and 31.6 ppm (194.5 μ M) nicotine slightly promoted egg production by ~4% compared to the control ($p < 0.05$), however this is unlikely to be biologically significant. Although the entire brood sizes were not compared, up to one-fifth of eggs can be produced after 72 h of adulthood. Taken together, chronic exposure to micromolar concentrations of nicotine promotes early egg-laying events and slightly increases eggs production during the first 72 h of adulthood.

We also investigated the expression patterns of 18 protein-coding genes belonging to different gene families. While many of these selected genes are known to regulate functions including egg-laying and stress response [11,24,25], little is known whether these genes are targets of nicotine and how nicotine affects expressions of these gene. For example, *egl* family genes *egl-10* and *egl-44* exhibited significant changes. Most *egl* genes were identified in screening of egg-laying defective mutants; their functions are associated with the formation of transcription factors, transmembrane G-protein-coupled receptors, and modulating motor neuron functions related to egg-laying [25,26]. We found that at 1 ppm nicotine, *egl-44* exhibited a significant 5.6-fold up-regulation, suggesting *egl-44* are among those early response genes sensitive to low dose nicotine exposure.

Egl-10 is highly conserved with mammalian regulator of G protein signaling (RGS) genes, which regulate G-protein signaling pathways and modulate frequencies of periodic behaviors. While loss-of-function mutant, overexpression of *egl-10*, and wild-type all produce similar number of eggs, the *egl-10* overexpressers laid eggs more frequently than the wild-type [27]. Although *C. elegans* egg-laying is a type of periodic behavior, the regulation of *egl-10* by nicotine has never been reported before. The eggs of *C. elegans* are laid in clustered episodes with intervals between the events [12]. It was reported that chronic exposure to 30 mM of nicotine for 16 h in NGM medium results in abnormality of this periodic behavior; the egg-laying cluster is shortened and the intercluster time interval is longer [13,14]. In this study we found that *egl-10* exhibited 22.7-fold up-regulation under 3.16 ppm (19.5 μ M) nicotine exposure. This suggests that *egl-10* may play a key role in regulating the periodic egg-laying behaviors in response to nicotine exposure.

The other gene, *egl-44*, is mostly upregulated. *Egl-44* encodes a putative transcription regulatory protein similar to the transcription enhancer factor (TEF) protein in vertebrates. It functions to promote the differentiation and migration of HSN motor neurons needed for egg-laying [25] and to prevent excess dendritic branching of the PVD neurons [28]. The *hlh-14* also regulates differentiations and functions of HSN [29]. *Hlh-14* was the most upregulated gene detected in this study under chronic treatment of 3.16 ppm nicotine. Taken together, the nicotine-induced aberrant expressions of *egl-10*, *egl-44*, and *hlh-14* indicates an adaptive response to maintain the normal egg-laying activities during low nicotine exposure.

Given the primary target of nicotine is the nicotinic acetylcholine receptor (nAChR), it is not surprising to see that several cholinergic signaling-related genes were changed by chronic low nicotine exposure. The K^+ channel gene *unc-103* expresses in HSN neurons and vulval muscles, regulating acetylcholine-mediated muscle excitability and egg-laying behaviors. The loss-of-function *unc-103* mutant hermaphrodites reduced egg-laying behaviors and laid embryos prematurely, while a supply of *unc-103* mRNA to vulval muscle restored the egg-laying inhibition effects in M9 buffer [30]. The gene *unc-50* encodes a Golgi protein required for traffic-king of assembled nAChRs to the plasma membrane in *C. elegans* [31]. In addition, the nicotine-induced gene *ric-3* is also involved in the trafficking and assembly

of several nicotinic acetylcholine receptors [32,33]. Interestingly, *unc-50* and *ric-3* exhibited similar trend in gene expression in this study, suggesting the trafficking and assembling of functional nicotinic receptor were subjected to regulation by nicotine exposure. The ryanodine receptor gene *unc-68* regulates the coupling of excitation–contraction of body-wall muscle, required for normal locomotive behaviors [34,35]. All three *unc* genes and *ric-3* are up-regulated >10-fold at the 3.16 ppm dose, suggesting active regulation of excitatory neuronal functions by the AChR agonist nicotine.

As nicotine induces oxidative stress and DNA damage in mammals [4,36], we tested if the oxidative stress-responsive genes are changed in *C. elegans* following nicotine exposure. We found that *oxi-1* was significantly up-regulated while *sod-1* and *old-1* were in general down-regulated. This indicated nicotine may affect the stress-defense system itself, besides causing oxidative stress. *Oxi-1* encodes a family of proteins homologous to human ubiquitin-protein ligase [24], suggesting the level ubiquitination may be increased under nicotine exposure. On the other hand, the down-regulation of *sod-1* and *old-1* induced by nicotine may result in more oxidative injuries and life-span defects in exposed animals. More studies need to be performed to investigate nicotine-related oxidative stress and associated DNA damage in *C. elegans*.

Although previous studies have investigated functions of many tested genes, this study identified genes affected by chronic micromolar range nicotine exposure. Some genes were identified as novel targets of nicotine that are important players involved in neuronal development, egg-laying, nAChR functions, oxidative stress and aging. Some of the nicotine-induced genes have orthologs in vertebrates and humans, including *egl-10*, *egl-44*, *unc-103*, *unc-50*, *unc-68*, *oxi-1*, and *old-1*. Novel gene targets identified in this study may provide new mechanistic insights into nicotine toxicity in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The work was partially supported by the National Institutes of Health (NIH) Grant R03DA032515 from the National Institute on Drug Abuse (NIDA). The content is solely the responsibility of the authors and does not necessarily represent the official views of NIDA. We also thank supports from the Undergraduate Biotechnology Research Fellowship program of the North Carolina Biotechnology Center (NCBC).

References

1. Dani JA, Bertrand D. Nicotinic acetylcholine receptors and nicotinic cholinergic mechanisms of the central nervous system. *Annual Review of Pharmacology and Toxicology*. 2007; 47:699–729.
2. Pidoplichko VI, Noguchi J, Areola OO, Liang Y, Peterson J, Zhang TX, et al. Nicotinic cholinergic synaptic mechanisms in the ventral tegmental area contribute to nicotine addiction. *Learning & Memory*. 2004; 11:60–9. [PubMed: 14747518]
3. Besson M, Granon S, Mameli-Engvall M, Cloez-Tayarani I, Maubourguet N, Cormier A, et al. Long-term effects of chronic nicotine exposure on brain nicotinic receptors. *Proceedings of the National Academy of Sciences of the United States of America*. 2007; 104:8155–60. [PubMed: 17470777]
4. Babizhayev MA, Savel'yeva EL, Moskvina SN, Yegorov YE. Telomere length is a biomarker of cumulative oxidative stress, biologic age, and an independent predictor of survival and therapeutic treatment requirement associated with smoking behavior. *American Journal of Therapeutics*. 2010; 18:E209–26. [PubMed: 20228673]

5. Torres LHL, Moreira WL, Garcia RCT, Annoni R, Carvalho ALN, Teixeira SA, et al. Environmental tobacco smoke induces oxidative stress in distinct brain regions of infant mice. *Journal of Toxicology and Environmental Health-Part a-Current Issues*. 2012; 75:971–80.
6. Rogers JM. Tobacco and pregnancy. *Reproductive Toxicology*. 2009; 28:152–60. [PubMed: 19450949]
7. Hatsukami DK, Stead LF, Gupta PC. Tobacco addiction. *Lancet*. 2008; 371:2027–38. [PubMed: 18555914]
8. Schafer WR. Genetic analysis of nicotinic signaling in worms and flies. *Journal of Neurobiology*. 2002; 53:535–41.
9. Feng ZY, Li W, Ward A, Piggott BJ, Larkspur ER, Sternberg PW, et al. A *C. elegans* model of nicotine-dependent behavior: regulation by TRP-family channels. *Cell*. 2006; 127:621–33. [PubMed: 17081982]
10. Vella MC, Slack FJ. *C. elegans* MicroRNAs. 2005
11. Kim J, Poole DS, Waggoner LE, Kempf A, Ramirez DS, Treschow PA, et al. Genes affecting the activity of nicotinic receptors involved in *Caenorhabditis elegans* egg-laying behavior. *Genetics*. 2001; 157:1599–610. [PubMed: 11290716]
12. Wei Geng PC, Megan P, Schafer WR. *Caenorhabditis elegans* egg-laying detection and behavior study using image analysis. *Journal on Applied Signal Processing*. 2005; 14:2229–40.
13. Matta SG, Balfour DJ, Benowitz NL, Boyd RT, Buccafusco JJ, Caggiula AR, et al. Guidelines on nicotine dose selection for in vivo research. *Psychopharmacology*. 2007; 190:269–319. [PubMed: 16896961]
14. Waggoner LE, Dickinson KA, Poole DS, Tabuse Y, Miwa J, Schafer WR. Long-term nicotine adaptation in *Caenorhabditis elegans* involves PKC-dependent changes in nicotinic receptor abundance. *Journal of Neuroscience*. 2000; 20:8802–11.
15. Sobkowiak R, Kowalski M, Lesicki A. Concentration- and time-dependent behavioral changes in *Caenorhabditis elegans* after exposure to nicotine. *Pharmacology Biochemistry and Behavior*. 2011; 99:365–70.
16. Schafer WF. Genetics of egg-laying in worms. *Annual Review of Genetics*. 2006; 40:487–509.
17. Brenner S. The genetics of *Caenorhabditis elegans*. *Genetics*. 1974; 77:71–94. [PubMed: 4366476]
18. Roh JY, Park YJ, Choi J. A cadmium toxicity assay using stress responsive *Caenorhabditis elegans* mutant strains. *Environmental Toxicology and Pharmacology*. 2009; 28:409–13. [PubMed: 21784035]
19. Zhang Y, Chen D, Smith MA, Zhang B, Pan X. Selection of reliable reference genes in *Caenorhabditis elegans* for analysis of nanotoxicity. *PLoS ONE*. 2012; 7:e31849. [PubMed: 22438870]
20. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{−(Delta Delta C(T))} method. *Methods*. 2001; 25:402–8. [PubMed: 11846609]
21. Pidoplichko VI, DeBiasi M, Williams JT, Dani JA. Nicotine activates and desensitizes midbrain dopamine neurons. *Nature*. 1997; 390:401–4. [PubMed: 9389479]
22. Benowitz NL, Kuyt F, Jacob P. Circadian blood nicotine concentrations during cigarette-smoking. *Clinical Pharmacology & Therapeutics*. 1982; 32:758–64. [PubMed: 7140139]
23. Altun, ZF.; Hall, DH. Introduction.. *WormAtlas*. 2009. <http://dx.doi.org/10.3908/wormatlas.1.1>
24. Yanase S, Ishi N. Cloning of the oxidative stress-responsive genes in *Caenorhabditis elegans*. *Journal of Radiation Research*. 1999; 40:39–47. [PubMed: 10408176]
25. Wu J, Duggan A, Chalfie M. Inhibition of touch cell fate by egl-44 and egl-46 in *C. elegans*. *Genes & Development*. 2001; 15:789–802. [PubMed: 11274062]
26. Moresco JJ, Koelle MR. Activation of EGL-47, a G alpha(o)-coupled receptor, inhibits function of hermaphrodite-specific motor neurons to regulate *Caenorhabditis elegans* egg-laying behavior. *Journal of Neuroscience*. 2004; 24:8522–30.
27. Koelle MR, Horvitz HR. EGL-10 regulates G protein signaling in the *C. elegans* nervous system and shares a conserved domain with many mammalian proteins. *Cell*. 1996; 84:115–25. [PubMed: 8548815]

28. Smith CJ, Watson JD, Spencer WC, O'Brien T, Cha B, Albeg A, et al. Time-lapse imaging and cell-specific expression profiling reveal dynamic branching and molecular determinants of a multi-dendritic nociceptor in *C. elegans*. *Developmental Biology*. 2010; 345:18–33. [PubMed: 20537990]
29. Frank CA, Baum PD, Garriga G. HLH-14 is a *C. elegans* achaete-scute protein that promotes neurogenesis through asymmetric cell division. *Development*. 2003; 130:6507–18. [PubMed: 14627726]
30. Reiner DJ, Weinshenker D, Tian H, Thomas JH, Nishiwaki K, Miwa J, et al. Behavioral genetics of *Caenorhabditis elegans* UNC-103-encoded ERG-like K⁺ channel. *Journal of Neurogenetics*. 2006; 20:41–66. [PubMed: 16807195]
31. Eimer S, Gottschalk A, Hengartner M, Horvitz HR, Richmond J, Schafer WR, et al. Regulation of nicotinic receptor trafficking by the transmembrane Golgi protein UNC-50. *EMBO Journal*. 2007; 26:4313–23. [PubMed: 17853888]
32. Shteingauz A, Cohen E, Biala Y, Treinin M. The BTB-MATH protein BATH-42 interacts with RIC-3 to regulate maturation of nicotinic acetylcholine receptors. *Journal of Cell Science*. 2009; 122:807–12.
33. Williams ME, Burton B, Urrutia A, Shcherbatko A, Chavez-Noriega LE, Cohen CJ, et al. Ric-3 promotes functional expression of the nicotinic acetylcholine receptor alpha 7 subunit in mammalian cells. *Journal of Biological Chemistry*. 2005; 280:1257–63. [PubMed: 15504725]
34. Liu Q, Chen BJ, Yankova M, Morest DK, Maryon E, Hand AR, et al. Presynaptic ryanodine receptors are required for normal quantal size at the *Caenorhabditis elegans* neuromuscular junction. *Journal of Neuroscience*. 2005; 25:6745–54. [PubMed: 16033884]
35. Maryon EB, Coronado R, Anderson P. unc-68 encodes a ryanodine receptor involved in regulating *C. elegans* body-wall muscle contraction. *Journal of Cell Biology*. 1996; 134:885–93. [PubMed: 8769414]
36. Kahl VFS, Reyes JM, Sarmento MS, da Silva J. Mitigation by vitamin C of the genotoxic effects of nicotine in mice, assessed by the comet assay and micronucleus induction. *Mutation Research-Genetic Toxicology and Environmental Muta-genesis*. 2012; 744:140–4.
37. Hardaker LA, Singer E, Kerr R, Zhou GT, Schafer WR. Serotonin modulates locomotory behavior and coordinates egg-laying and movement in *Caenorhabditis elegans*. *Journal of Neurobiology*. 2001; 49:303–13. [PubMed: 11745666]
38. Chase DL, Pepper JS, Koelle MR. Mechanism of extrasynaptic dopamine signaling in *Caenorhabditis elegans*. *Nature Neuroscience*. 2004; 7:1096–103.
39. van der Linden AM, Simmer F, Cuppen E, Plasterk RH. The G-protein beta-subunit GPB-2 in *Caenorhabditis elegans* regulates the G(o)alpha-G(q)alpha signaling network through interactions with the regulator of G-protein signaling proteins EGL-10 and EAT-16. *Genetics*. 2001; 158:221–35. [PubMed: 11333232]
40. Esposito G, Amoroso MR, Bergamasco C, Di Schiavi E, Bazzicalupo P. The G protein regulators EGL-10 and EAT-16, the G(i)alpha GOA-1 and the G(q)alpha EGL-30 modulate the response of the *C. elegans* ASH polymodal nociceptive sensory neurons to repellents. *BMC Biology*. 2010; 8. [PubMed: 20102595]
41. Frokjaer-Jensen C, Kindt KS, Kerr RA, Suzuki H, Melnik-Martinez K, Gerstbreih B, et al. Effects of voltage-gated calcium channel subunit genes on calcium influx in cultured *C. elegans* mechanosensory neurons. *Journal of Neurobiology*. 2006; 66:1125–39.
42. Shtonda B, Avery L. CCA-1, EGL-19 and EXP-2 currents shape action potentials in the *Caenorhabditis elegans* pharynx. *Journal of Experimental Biology*. 2005; 208:2177–90. [PubMed: 15914661]
43. Jospin M, Jacquemond V, Mariol MC, Segalat L, Allard B. The L-type voltage-dependent Ca²⁺ channel EGL-19 controls body wall muscle, function in *Caenorhabditis elegans*. *Journal of Cell Biology*. 2002; 159:337–47.
44. Mitani S, Du HP, Hall DH, Driscoll M, Chalfie M. Combinatorial control of touch receptor neuron expression in *Caenorhabditis elegans*. *Development*. 1993; 119:773–83. [PubMed: 8187641]

45. Moresco JJ, Koelle MR. Activation of EGL-47, a G α (o)-coupled receptor, inhibits function of hermaphrodite-specific motor neurons to regulate *Caenorhabditis elegans* egg-laying behavior. *Journal of Neuroscience*. 2004; 24:8522–30. [PubMed: 15456826]
46. Nicholas HR, Hodgkin J. The *C. elegans* Hox gene *egl-5* is required for correct development of the hermaphrodite hindgut and for the response to rectal infection by *Microbacterium nematophilum*. *Developmental Biology*. 2009; 329:16–24. [PubMed: 19232338]
47. Kalis AK, Murphy MW, Zarkower D. EGL-5/ABD-B plays an instructive role in male cell fate determination in the *C. elegans* somatic gonad. *Developmental Biology*. 2010; 344:827–35. [PubMed: 20553900]
48. Irazoqui JE, Ng A, Xavier RJ, Ausubel FM. Role for beta-catenin and HOX transcription factors in *Caenorhabditis elegans* and mammalian host epithelial-pathogen interactions. *Proceedings of the National Academy of Sciences of the United States of America*. 2008; 105:17469–74. [PubMed: 18981407]
49. Singhvi A, Frank CA, Garriga G. The T-Box gene *tbx-2*, the homeobox gene *egl-5* and the asymmetric cell division gene *ham-1* specify neural fate in the HSN/PHB lineage. *Genetics*. 2008; 179:887–98. [PubMed: 18505863]
50. Chisholm A. Control of cell fate in the tail region of *C. elegans* by the gene *Egl-5*. *Development*. 1991; 111:921–32. [PubMed: 1879361]
51. Poole RJ, Bashllari E, Cochella L, Flowers EB, Hobert O. A genome-wide RNAi screen for factors involved in neuronal specification in *Caenorhabditis elegans*. *Plos Genetics*. 2011;7.
52. Clark SG, Chisholm AD, Horvitz HR. Control of cell fates in the central body region of *C. elegans* by the homeobox gene *lin-39*. *Cell*. 1993; 74:43–55. [PubMed: 8101475]
53. Pellegrino MW, Farooqui S, Frohli E, Rehrauer H, Kaeser-Pebarnard S, Muller F, et al. LIN-39 and the EGFR/RAS/MAPK pathway regulate *C. elegans* vulval morphogenesis via the VAB-23 zinc finger protein. *Development*. 2011; 138:4649–60. [PubMed: 21989912]
54. Wagmaister JA, Miley GR, Morris CA, Gleason JE, Miller LM, Kornfeld K, et al. Identification of cis-regulatory elements from the *C. elegans* Hox gene *lin-39* required for embryonic expression and for regulation by the transcription factors LIN-1, LIN-31 and LIN-39. *Developmental Biology*. 2006; 297:550–65. [PubMed: 16782085]
55. Shemer G, Podbilewicz B. LIN-39/Hox triggers cell division and represses EFF-1 fusogen-dependent vulva cell fusion. *Genes & Development*. 2002; 16:3136–41.
56. Millar NS. RIC-3: a nicotinic acetylcholine receptor chaperone. *British Journal of Pharmacology*. 2008; 153:S177–83. [PubMed: 18246096]
57. Halevi S, McKay J, Palfreyman M, Yassin L, Eshel M, Jorgensen E, et al. The *C. elegans* *ric-3* gene is required for maturation of nicotinic acetylcholine receptors. *EMBO Journal*. 2002; 21:1012–20.
58. Zhang YQ, Chen DL, Ennis AC, Polli JR, Xiao P, Zhang BH, et al. Chemical dispersant potentiates crude oil impacts on growth, reproduction, and gene expression in *Caenorhabditis elegans*. *Archives of Toxicology*. 2013; 87:371–82.
59. Garcia LR, Sternberg PW. *Caenorhabditis elegans* UNC-103 ERG-like potassium channel regulates contractile behaviors of sex muscles in males before and during mating. *Journal of Neuroscience*. 2003; 23:2696–705.
60. Reiner DJ, Newton EM, Tian H, Thomas JH. Diverse behavioural defects caused by mutations in *Caenorhabditis elegans* *unc-43* CaM kinase II. *Nature*. 1999; 402:199–203. [PubMed: 10647014]
61. Pietsch K, Saul N, Menzel R, Sturzenbaum SR, Steinberg CEW. Quercetin mediated lifespan extension in *Caenorhabditis elegans* is modulated by *age-1*, *daf-2*, *sek-1* and *unc-43*. *Biogerontology*. 2009; 10:565–78. [PubMed: 19043800]
62. Sagasti A, Hisamoto N, Hyodo J, Tanaka-Hino M, Matsumoto K, Bargmann CI. The CaMKII UNC-43 activates the MAPKKK NSY-1 to execute a lateral signaling decision required for asymmetric olfactory neuron fates. *Cell*. 2001; 105:221–32. [PubMed: 11336672]
63. Boulon T, Fauvin A, Charvet CL, Cortet J, Cabaret J, Bessereau JL, et al. Functional reconstitution of *Haemonchus contortus* acetylcholine receptors in *Xenopus* oocytes provides mechanistic insights into levamisole resistance. *British Journal of Pharmacology*. 2011; 164:1421–32. [PubMed: 21486278]

64. Adachi R, Kagawa H. Genetic analysis of ryanodine receptor function in *Caenorhabditis elegans* based on unc-68 revertants. *Molecular Genetics and Genomics*. 2003; 269:797–806.
65. Doonan R, McElwee JJ, Matthijssens F, Walker GA, Houthoofd K, Back P, et al. Against the oxidative damage theory of aging: superoxide dismutases protect against oxidative stress but have little or no effect on life span in *Caenorhabditis elegans*. *Genes Development*. 2008; 22:3236–41.
66. Shibata Y, Branicky R, Landaverde IO, Hekimi S. Redox regulation of germline and vulval development in *Caenorhabditis elegans*. *Science*. 2003; 302:1779–82.
67. Samann J, Hegermann J, von Gromoff E, Eimer S, Baumeister R, Schmidt E. *Caenorhabditis elegans* LRK-1 and PINK-1 act antagonistically in stress response and neurite outgrowth. *Journal of Biological Chemistry*. 2009; 284:16482–91. [PubMed: 19251702]
68. Camon E, Magrane M, Barrell D, Binns D, Fleischmann W, Kersey P, et al. The gene ontology annotation (GOA) project: implementation of GO in SWISS-PROT, TrEMBL, and InterPro. *Genome Research*. 2003; 13:662–72. [PubMed: 12654719]
69. Ayyadevara S, Tazearslan C, Bharill P, Alla R, Siegel E, Shmookler Reis RJ. *Caenorhabditis elegans* PI3K mutants reveal novel genes underlying exceptional stress resistance and lifespan. *Aging Cell*. 2009; 8:706–25. [PubMed: 19764929]
70. Reis RJS, Ayyadevara S, Crow WA, Lee T, Delongchamp RR. Gene categories differentially expressed in *C. elegans* Age-1 mutants of extraordinary longevity: new insights from novel data-mining procedures. *Journals of Gerontology Series a-Biological Sciences and Medical Sciences*. 2012; 67:366–75.
71. Murakami S, Johnson TE. The OLD-1 positive regulator of longevity and stress resistance is under DAF-16 regulation in *Caenorhabditis elegans*. *Current Biology*. 2001; 11:1517–23. [PubMed: 11591319]
72. Johnson TE, Henderson S, Murakami S, de Castro E, de Castro SH, Cypser J, et al. Longevity genes in the nematode *Caenorhabditis elegans* also mediate increased resistance to stress and prevent disease. *Journal of Inherited Metabolic Disease*. 2002; 25:197–206. [PubMed: 12137228]

Fig. 1.

Percentage no response to touch versus nicotine dosage. L3 *C. elegans* were dosed with different concentrations of nicotine in K-medium with food for 24 h and then subjected to gentle touch stimulus if not moving. Around 50 worms were placed in each well in triplicate for one biological replicate. Four biological replicates (four independent experiments) were performed totaling ~600 worms per dose. For each dose, the average “No response to touch” % of the 3 technical replicates was treated as one data point. This figure shows the mean and standard error (SE) of “No response to touch”% of 4 biological replicates ($n = 4$). Statistical analysis was performed by the ANOVA. Different letters indicated distinct statistical groups at $p < 0.05$ level.

Fig. 2.

Effects of different doses of nicotine (ppm) on the 24-h egg-laying pattern over 72 h post-dosing. L3 *C. elegans* were dosed for 24 h and then placed individually in NGM agar well with OP50 to allow egg-laying. Eggs were counted at three time points: 24, 48, and 72 h after dosing. Data at the time point 24 h represent the number of egg laid during the period of 0–24 h post-dosing. Data at the time point 48 h represent the number of egg laid during the period of 24–48 h post-dosing. Data at the time point 72 h represent the number of egg laid during the period of 48–72 h post-dosing. Error bars indicate the standard error (SE). Different symbols of the same type represent different statistical groups comparing at the same time point by repeated measures ANOVA at $p < 0.05$ level.

Fig. 3.

Effects of different doses of nicotine (ppm) on eggs production over 72 h post-dosing. L3 *C. elegans* were dosed for 24 h and then placed individually in NGM agar well with OP50 to allow egg-laying. Eggs were counted at three time points: 24, 48, and 72 h after dosing. Data at the time point 24 h represent the number of egg laid during the period of 0–24 h post-dosing. Data at the time point 48 h represent the number of egg laid during the period of 0–48 h post-dosing. Data at the time point 72 h represent the number of egg laid during the period of 0–72 h post-dosing. Error bars indicate the standard error (SE). Different symbols of the same type represent different statistical groups compared at the same time point by repeated measures ANOVA at $p < 0.05$ level.

Fig. 4.

Expression fold change of the 18 protein-coding genes. L3 *C. elegans* were dosed for 24 h in K-medium and then RNAs were extracted for gene expression analysis. Fold changes of genes were normalized using Y45F10D.4 mRNA and are presented as relative units compared to control (control = 1, above 1 mean up-regulated, below 1 means down-regulated). Y-axis uses logarithmic scale and shows the mean and standard deviation (SD) of fold changes of 3 independent experiments for individual genes ($n = 3$).

Table 1

Functional classification of the eighteen tested genes.

Gene symbol	Locus tag	Gene description	Egg-laying/reproduction	Develop/cell fate	Muscle and neuron	Stress response/signaling	Life span	References
cat-4	F32G8.6	abnormal CATecholamine distribution						[37]
egl-10	F28C1.2	Egg laying defective						[38-40]
egl-19	C48A7.1	Egg Laying defective						[41-43]
egl-44	F28B12.2	Egg Laying defective						[25,44]
egl-47	C50H2.2	Egg Laying defective						[45]
egl-5	C08C3.1	Egg Laying defective						[46-50]
hlh-14	C18A3.8	Helix Loop Helix						[29,51]
lin-39	C07H6.7	abnormal cell L1Neage						[52-55]
ric-3	T14A8.1	Resistance to Inhibitors of Cholinesterase						[32,56-58]
unc-103	C30D11.1	UNCordinated						[30,59]
unc-43	K11E8.1	UNCordinated						[60-62]
unc-50	T07A5.2	UNCordinated						[31,63]
unc-68	K11C4.5	UNCordinated						[34,35,64]
sod-1	C15F1.7	SOD (superoxide dismutase)						[58,65,66]
pink-1	EEED8.9	PINK (PTEN-Induced Kinase) homolog						[67]
oxi-1	Y39A1C.2	Oxidative stress Induced						[24,68]
age-1	B0334.8	AGEing alteration						[69,70]
old-1	C08H9.5	Overexpression Longevity Determinant						[71,72]