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Drug-Dependent Behaviors and Nicotinic Acetylcholine Receptor Expressions in *Caenorhabditis elegans* Following Chronic Nicotine Exposure

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Abstract

Nicotine, the major psychoactive compound in tobacco, targets nicotinic acetylcholine receptors (nAChRs) and results in drug dependence. The nematode *Caenorhabditis elegans'* (*C. elegans*) genome encodes conserved and extensive nicotinic receptor subunits, representing a useful system to investigate nicotine-induced nAChR expressions in the context of drug dependence. However, the *in vivo* expression pattern of nAChR genes under chronic nicotine exposure has not been fully investigated. To define the role of nAChR genes involved in nicotine-induced locomotion changes and the development of tolerance to these effects, we characterized the locomotion behavior combining the use of two systems: the Worm Tracker hardware and the WormLab software. Our results indicate that the combined system is an advantageous alternative to define drug-dependent locomotion behavior in *C. elegans*. Chronic (24-hour dosing) nicotine exposure at 6.17 and 61.7 μM induced nicotine-dependent behaviors, including drug stimulation, tolerance/adaption, and withdrawal responses. Specifically, the movement speed of naïve worms on nicotine-containing environments was significantly higher than on nicotine-free environments, suggesting locomotion stimulation by nicotine. In contrast, the 24-hour 6.17 μM nicotine-treated worms exhibited significantly higher speeds on nicotine-free plates than on nicotine-containing plates. Furthermore significantly increased locomotion behavior during nicotine cessation was observed in worms treated with a higher nicotine concentration of 61.7 μM. The relatively low locomotion speed of nicotine-treated worms on nicotine-containing environments also indicates adaption/tolerance of worms to nicotine following chronic nicotine exposure. In addition, this study provides useful information regarding the comprehensive *in vivo* expression profile of the 28 "core" nAChRs following different dosages of chronic nicotine treatments. Eleven genes (*lev-1*, *acr-6*, *acr-7*, *acr-11*, *lev-8*, *acr-14*, *acr-16*, *acr-20*, *acr-21*, *ric-3*, and *unc-29*) were significantly up-regulated

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following 61.7 μM nicotine treatment, in which worms showed significantly increased locomotion behavior. This study provides insights into the linkage between nicotine-induced locomotion behavior and the regulation of nicotinic acetylcholine receptors.

Keywords

nicotine; drug-dependent behaviors; nAChRs; gene expression; *C. elegans*

Introduction

Drug addiction has become a worldwide health issue with tobacco use being the leading cause of preventable deaths (U.S. Department of Health and Human Services, 2014). Nicotine, the major psychoactive compound in tobacco, primarily targets the nicotinic acetylcholine receptors (nAChRs). nAChRs are expressed at the synapse of nerve and muscle cells and are responsible for mediating excitatory synaptic transmission at the neuromuscular junctions and in the nervous system (Jones & Sattelle, 2003). One mechanism of the development of drug-dependence behaviors is that the activation of nAChRs on dopaminergic neurons by the endogenous ligand acetylcholine (ACh) or exogenous agonists, such as nicotine, stimulates dopamine release, mediating rewarding effects of nicotine (Cahir *et al.*, 2011; Marks, 2013). Nicotine administration results in behavioral stimulation after acute exposure, behavioral and physiological tolerance after chronic exposure, and dependence/withdrawal symptoms upon nicotine removal (Feng et al., 2006; Mineur & Picciotto, 2008).

The nAChRs have been characterized under a dicysteine loop (CC-loop) superfamily of ligand-gated ion channels. Each nAChR is a pentameric membrane protein, consisting of five subunits surrounding a central cation-permeable pore (Jones *et al.*, 2007). Subunits are classified into two major categories: α or non-α subunits. The α subunits have an adjacent cysteine-cysteine motif at the ACh binding site, while non-α subunits do not have this motif (Changeux & Edelstein, 1998). The composition of each nAChR can be homomeric or heteromeric, leading to diversity in receptor function and pharmacology (Gerzanich *et al.*, 1998; Towers *et al.*, 2005). Sequence comparisons reveal that nAChR subunits are highly conserved across species (Mongan *et al.*, 1998; Karlin, 2002; Towers *et al.*, 2005). For example, the neuronal homomer-forming α7 subunits are one of the most abundant and widespread subtypes in the vertebrate brain (Mineur & Picciotto, 2008). On the other hand, α7 and β2 are the primary nAChR subunits expressed in vertebrate nervous systems and form the heteromeric nicotinic receptors (Azam *et al.*, 2003). These receptors require proper folding and trafficking provided by the chaperone protein RIC-3 (Halevi *et al.*, 2002; Millar, 2008). RIC-3 enables α7 subunits to properly fold into the correct configuration and then be exported out of the endoplasmic reticulum (Halevi *et al.*, 2002; Lansdell *et al.*, 2005). RIC-3 predominately interacts with the α7 subunits to form homomeric receptors. However, there has been evidence that RIC-3 provides assistance to form a few heteromeric receptors (Castillo *et al.*, 2005). In the nematode *Caenorhabditis elegans* (*C. elegans*), ACR-16-like group resembles the vertebrate α7 subunit group and contains the second-highest number of nAChR subunit genes after *Fugu rubripes* (Jones *et al.*, 2007).

The *C. elegans* is an excellent model organism to address genetic mechanisms associated with neurological behavioral changes due to its fully sequenced genome and well-characterized nervous system (Feng *et al.*, 2006; Cutter *et al.*, 2009). The life cycle of wide-type *C. elegans* is ~ 3 days and life span is ~ 3 weeks at 20 °C (Wormatlas), shorter than most vertebrate model organisms. The nervous system of *C. elegans* adult hermaphrodites' consists of 302 neurons, whose identity, location, lineage, and synaptic connectivity have been well-characterized (Schafer, 2004). The *C. elegans* has been a popular choice of model organism in neuro-behavioral studies in response to toxicants. Previous studies have examined *C. elegans* behavior changes in parameters such as head thrashing, locomotion, mortality, egg-laying, Ω/U-turns, chemotaxis, and pharyngeal pumping (Riddle *et al.*, 1997; Anderson *et al.*, 2001; Anderson *et al.*, 2004; Feng *et al.*, 2006; Sobkowiak *et al.*, 2011; Rose *et al.*, 2013). The genome of *C. elegans* has been completely sequenced and shows a high level of conservation with vertebrates (Cutter et al., 2009). Importantly, the *C. elegans* genome encodes a diverse nAChR gene family that consists of at least 28 authentic nicotinic receptor subunit homologs, representing a useful system to investigate nicotine-induced cholinergic transmission and signaling (Schafer, 2004). Table 1 details the description of the 28 nAChRs. Based on sequence homology, these 28 nAChRs can be classified into five "core" groups: ACR-16-like, UNC-29-like, UNC-38-like, DEG-3-like, and ACR-8-like (Jones & Sattelle, 2003). The nicotine-sensitive ACR-16-like group closely resembles vertebrate α7 nAChR subunits, the UNC-29-like and UNC-38-like group members are close homologs to *Drosophila* and vertebrate non- α subunits (Jones & Sattelle, 2003). The DEG-3-like and ACR-8-like groups are uniquely expressed in nematodes (Jones & Sattelle, 2003). Despite previous works revealing functions of individual nAChRs, their *in vivo* expression pattern under chronic nicotine exposure has not been fully investigated.

Feng *et al.* first defined nicotine-dependent behaviors in *C. elegans* using the Wormtracker system and a Worm analyzer software developed by the Schafer and Sternberg lab (Feng *et al.*, 2006). Naïve worms displayed a "locomotion-stimulation" phase after being transferred to a nicotine-containing environment. The stimulation was evident by increased movement speed during the 4–16 minute period after exposure to nicotine, whereas locomotion speed was decreased during the same time period when naïve worms were placed in nicotine-free environment. Feng *et al.* also demonstrated that worms subjected to chronic nicotine treatment (16-hour) developed nicotine adaption/tolerance. Chronic nicotine-treated *C. elegans* did not exhibit the "locomotion-stimulation" phase after being transferred to nicotine-containing plates; instead they showed a naïve-like behavior (low speed) on nicotine plates, indicating nicotine adaption/tolerance. Significantly, nicotine withdrawal symptoms were demonstrated as the nicotine-treated worms displayed abnormally high locomotion speed in nicotine-deprived environments (Feng *et al.*, 2006).

Using the Parallel Worm Tracking system (Ramot *et al.*, 2008), Sobkowiak *et al.* also examined the locomotion speeds of *C. elegans* exposed to various concentrations of nicotine (Sobkowiak *et al.*, 2011). They found that *C. elegans* locomotion significantly increased as the concentration of nicotine increased from 0.001 mM to 0.1 mM and then decreased from 0.1 mM to 30 mM (Sobkowiak *et al.*, 2011). Sobkowiak *et al.* also determined the longest stimulation effect lasted for 70 minutes in 0.1 mM treated worms. Although worms were

stimulated by nicotine concentrations of 0.001–1 mM, inhibition of locomotion was also observed. At high concentrations of nicotine (30 mM), nearly all worms stopped moving after 10 minutes. Another study by Rose *et al.* found that *C. elegans* exposed to 30 μM of nicotine from zygote stage and the zygote + larval stages displayed a greater number of reversal movements when tested on nicotine free agar when compared to the control (Rose *et al.*, 2013). However, the larval-exposed group was the only group to display spontaneous reversals greater than the control worms when tested on nicotine containing agar (Rose *et al.*, 2013).

The Worm Tracker settings used in the Feng *et al.*'s study required a self-assembled moving microscope stage to track behaviors of a single worm at one time and free software named the Worm Analyzer for video analysis. However, it is not known if the commercially available software, Wormlab, will be a sufficient alternative to characterize nicotine associated locomotion behavior. The dual purposes of this study were to characterize chronic nicotine exposure-induced locomotion behavior using a combined Worm Tracker hardware and the WormLab software system and to detect related nAChRs gene expression using quantitative real-time PCR (qRT-PCR).

Materials & Methods

C. elegans cultivation and treatment

The wild type strain Bristol N2 of *C. elegans*, retrieved from the *Caenorhabditis* Genetics Center, was cultivated on nematode growth medium (NGM) seeded with *Escherichia coli* (*E. coli*) strain OP50 as a source of food according to a standard protocol (Brenner, 1974). All experiments used age synchronized worms at larval stage 3 (L3), 30 hour after hatching at 20°C (Roh JY, 2009).

L-nicotine (98% pure solution) was obtained from ACROS Organics. Nicotine dosing solutions were diluted with K-medium (0.032 M KCl and 0.051 M NaCl) to reach concentrations of 6.17 μM and 61.7 μM. These two concentrations were considered "effective dosages", as previous reports revealed that dosages in this range promote early egg-laying (Smith Jr *et al.*, 2013) and cause locomotion stimulation (Sobkowiak *et al.*, 2011). A K-medium solution was used as a vehicle control. Worms were allotted to their respected nicotine dosages in liquid K-medium solutions or control solutions; *E. coli* OP50 was added as food, and the dosage tubes were then placed on a shaker (50 rpm) in a 20°C incubator for a 24 hour dosing period. A period of 24 hours exposure was deemed chronic because this is approximately 1/3 of the *C. elegans'* life cycle. Since nicotine is photosensitive, the stock solution was stored in a dark-brown container wrapped inside aluminum foil, the dosing solutions were freshly made and the exposures were performed in a dark incubator. The nicotine-containing NGM tracking plates were also freshly made and stored in dark refrigerator for less than one day until tracking.

C. elegans behavioral analysis

After 24 hours of nicotine exposure, worms were collected and rinsed with K-medium three times to remove residual nicotine and bacteria. Worms were then transferred to either

nicotine-containing (6 μM) or nicotine-free behavioral assay plates. The behavioral assay was conducted on 3 cm NGM agar plates seeded with a thin layer of *E. coli* OP50. Video recordings of the worms' behavior were performed using the hardware settings of Worm Tracker 2.0 (Schafer Lab, MRC LMB http://www.mrc-lmb.cam.ac.uk/wormtracker/). Six independent experiments were performed per dosage group. After allowing an acclimation time of 30 minutes on behavioral assay plates, five minute video recordings were taken by a DinoLite Pro AM413T Camera. All videos were analyzed using the MBF Science computer program WormLab 1.1.10. Figure 1 shows the viewing field of WormLab when the program was tracking the worms. Each worm was manually selected and then tracked. The locomotion speed, calculated by the Wormlab software as wavelength × oscillation frequency, were exported to Excel files and sorted into five speed ranges.

Impact of nicotine on nAChRs gene expression in C. elegans

After 24 hours of nicotine exposure, worms were centrifuged at 2000 rpm for 2 minutes. After removing the supernatant, worms were washed twice with M9 (0.042 M Na_2HPO_4, 0.022 M KH_2PO_4, 0.086 M NaCl, 0.001 M $MgSO_4{\cdot}7H_20$) and centrifuged again at 2000 rpm for 2 minutes to ensure excess nicotine and *E. coli* OP50 were removed. Immediately after the second rinse, the worm pellet was frozen in liquid nitrogen and stored at -80°C until analysis of nAChR genes. The expression levels of 29 protein-coding genes (28 nAChR genes and *ric-3*) were tested by the quantitative real-time polymerase chain reaction (qRT-PCR) method. The 28 nAChR genes were *lev-1, acr-2, acr-3, des-2, acr-5, acr-6, acr-7, acr-8, acr-9, acr-10, acr-11, acr-12, lev-8, acr-14, acr-15, acr-16, acr-17, acr-18, acr-19, acr-20, acr-21, deg-3, acr-23, acr-24, acr-25, unc-29, unc-38,* and *unc-63*.

Total RNA was extracted by the Ambion® *mir*Vana™ miRNA Isolation Kit by Life Technologies (Ambion, Austin, TX, USA). Total RNA was quantified by a Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE, USA). For reverse transcription (RT), 400 ng of total RNA from control, 6.17 μM, and 61.7 μM nicotine dosed worms was transcribed into single-stranded cDNA. RT was carried out in a 15 μL solution, which contained a calculated amount of nuclease-free water and 400 ng of total RNAs, 0.19 μL RNase Inhibitor (20U/μL), 0.15 μL 100 mM dNTPs (with dTTP), 10× RT Buffer, 1 μL MultiScribe™ Reverse Transcriptase (50U/μL), and 2 μL of Poly(T) Primer Mix. The reactions were performed with an Eppendorf Mastercycler ® Pro PCR machine using the following temperature program: initial stage of 16°C for 30 minutes followed by 42°C for 30 minutes and a final stage of 85°C for five minutes followed by a holding period at 4°C until samples can be stored at -20°C.

Quantitative RT-PCR (qPCR) analyses were conducted in a 20 μL solution, consisting of 7 μL nuclease-free water, 10 μL SYBR Green PCR Master Mix, 1 μL RT PCR product (diluted with 85 μL of nuclease-free water), and 2 μL of forward and reverse primers. A list of forward and reverse primer sequences for tested genes can be found in Table 2. The reactions were performed with an AppliedBiosystems 7300 Real Time PCR System programmed with a temperature setup as follows: initial enzyme activation stage of 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds.

All reactions had three technical replicates and each dose had four biological replicates, i.e. four independent experiments. In qPCR, F35G12.2, a conserved house-keeping gene that encodes a *C. elegans* ortholog of mitochondrial NAD+:isocitrate dehydrogenase, was employed as the reference gene for normalizing qPCR results (Taki & Zhang, 2013). The results were analyzed using the ΔΔCt method (Livak & Schmittgen, 2001).

Data Analysis

Statistical analysis was completed using IBM SPSS Statistics 20 software for Windows 7. The statistical test analysis of variance (ANOVA) determined if there were significant differences in locomotion speeds and gene expression fold changes between treatments groups. Two-way ANOVA/Univariate Analysis of Variance was performed to compare means in locomotion speeds using the speed as dependent variable, the dosage and the withdrawal condition (nicotine-free or nicotine-containing tracking) as two factors. If there was a significant difference at a $p < 0.05$ level, least significant difference (LSD) multiple comparisons were executed to compare means among different treatment groups. In addition, the chi-square statistical test was carried out using STATPerl to determine if there were significant differences (at a $p < 0.05$ level) in worm population distribution at different speed ranges between nicotine-free and nicotine-containing environments. If the output p value was expressed as $p = 0.000$ by the statistics software, it was reported as $p < 0.001$.

Results

Drug-dependent behavior characterization

The average locomotion speed of individual worms was calculated by the WormLab software. Two-way ANOVA analysis showed that there were significant differences among locomotion speeds in worms subjected to different dosages of nicotine treatments (0, 6.17, and 61.7 μM) when on either nicotine-free or nicotine-containing conditions (3 dosages × 2 conditions = 6 groups, df =5, $F = 126.5$, $p < 0.001$). After 30 minutes of acclimation, the average speed of naïve worms on nicotine-free plates was 10.1 ± 1.2 μm/sec ($n = 423$), whereas on nicotine-containing plates, the average speed was 194 ± 31 μm/sec ($n = 190$). The locomotion speed of naïve worms on nicotine-containing plates was significantly higher than on nicotine-free plates ($p < 0.001$, ANOVA). The pie charts on Figure 3A and 3B show the detailed percentage distributions of worms into five continuous speed ranges (0–20, 20–40, 40–80, 80–160, and > 160 μm/sec) according to their average locomotion speeds on either nicotine-free or nicotine-containing (6 μM) tracking plates. Worms on nicotine-containing plates displayed a maximum speed of 2092.2 μm/s, so the upper range of speed was set as 160 – 2100 μm/s (Figure 3). Since any speed of > 80 μm/sec was over two standard deviations (SDs) of the mean locomotion speed of 10.1 μm/sec, which was the speed of naïve worms on nicotine-free plates (naïve conditions), statistically 80 μm/sec can be considered as a threshold to distinguish normal and abnormal-high locomotion speeds. Most (98 %) naïve worms displayed a normal low speed of 0–80 μm/sec on nicotine-free plates. In contrast, 20% of naïve worms exhibited an abnormally high locomotion speed of > 80 μm/sec on nicotine-containing plates, as compared to only 2% on a nicotine-free environment ($p < 0.001$, chi-square analysis) (Figure 4).

Worms treated with a nicotine concentration of 6.17 μM in liquid medium for 24 hours showed behavioral characteristics of nicotine adaption and withdrawal symptoms. Figure 3C and 3D show the detailed percentage distributions of worms in this treatment into five continuous speed ranges according to their average locomotion speeds on either nicotine-free or nicotine-containing tracking plates. After 30 minutes of acclimation, the average speed of nicotine-treated worms on nicotine-free plates was 88.7 ± 6.4 μm/sec (n=387), whereas on nicotine-containing plates, the average speed was 51.9 ± 4.2 μm/sec (n = 251). The locomotion speed of nicotine-treated worms on nicotine-free plates was significantly higher than the worms on nicotine-containing plates ($p = 0.021$, ANOVA). Chi-square analysis also showed that a higher percentage of worms (35% versus 18%) exhibited high speeds of > 80 μm/sec when in nicotine-free versus nicotine-containing environments ($p < 0.001$, Figure 5). In addition, the 24-hour 6.17 μM nicotine-treated worms displayed significant ($p < 0.001$, ANOVA) lower average speeds (51.9 μm/sec) on nicotine-containing plates as compared to exposure of naïve worms on nicotine-containing plates (194 μm/sec). The nicotine-induced locomotion stimulation was significantly greater in worms treated with a higher nicotine concentration of 61.7 μM for 24 hrs. The average locomotion speed of the dosed worms on nicotine-free plates was as high as 640 ± 54 μm/sec (n = 64), significantly higher ($p < 0.001$, ANOVA) than those on nicotine-containing plates, which was 92.9 ± 54 μm/sec (n = 116). In nicotine-free environments, the mean locomotion speeds of 61.7 μM treated worms were also significantly higher than 6.17 μM treated worms ($p < 0.001$, ANOVA). Results from chi-square analysis were also consistent; a high percentage (91%) of worms were moving with a high speed of > 80 μm/sec on nicotine-free plates, which was significantly higher than 35% of worms with > 80 μm/sec on nicotine-containing plates ($p < 0.001$, chi-square analysis, Figure 6). Figure 7 depicts the locomotion speed stimulation as the dosage increased from 6.17 to 61.7 μM.

Expressions of nAChR genes

Since the direct target of nicotine is the nicotinic acetylcholine receptors (nAChRs), we tested the expressions of 28 nAChR genes under different nicotine treatments. Figure 8 shows the fold change in gene expression of all 28 tested nAChR genes as well as *ric-3*. Of these genes, thirteen were significantly up-regulated (*lev-1*, *acr-6*, *acr-7*, *acr-11*, *lev-8*, *acr-14*, *acr-15*, *acr-16*, *acr-20*, *acr-21*, *ric-3*, *unc-29*, and *unc-38*) and five were significantly down-regulated (*acr-2*, *acr-3*, *acr-5*, *acr-17*, and *unc-63*). Of the 13 up-regulated genes, five were at the 6.17 μM treatment group and eleven were at the 61.7 μM treatment group. The genes that were upregulated at both 6.17 and 61.7 μM treatments were *acr-11*, *acr-16* and *ric-3*. In contrast, genes that were significantly downregulated at both 6.17 and 61.7 μM treatments were *acr-2*, *acr-3*, *acr-5*, and *unc-63*. There were six genes (*lev-1*, *acr-2*, *acr-5*, *acr-6*, *acr-21*, and *unc-63*) that expressed > 1.5 fold regulation. Interestingly, the genes (*lev-1*, *acr-6*, and *acr-21*) that exhibited >1.5 fold up-regulation were all in the 61.7 μM treatment group, whereas those expressed > 1.5 fold down-regulation (*acr-2*, *acr-5*, and *unc-63*) were all in 6.17 μM treated worms. The *acr-5* gene was the most downregulated at both tested concentrations with 14.6 and 3.0 fold downregulation at 6.17and 61.7 μM groups, respectively.

Discussion

Nicotine exposure alters behaviors in *C. elegans* including pharyngeal pumping, egg-laying, and male mating behaviors (Matta et al., 2007). While high dosages of nicotine causes body paralysis/over-contraction of muscles and depressant-like symptoms on the nervous system, relatively low dosages results in locomotion stimulation following acute exposure in *C. elegans* (Benowitz, 1988; Sobkowiak *et al.*, 2011). Using the locomotion speed as a parameter, Feng *et al*. firstly define nicotine-dependent behaviors in *C. elegans*, which parallel vertebrate responses to nicotine (Feng *et al.*, 2006). We demonstrated in this study that an alternative behavioral testing system: the Worm Tracker hardware, without the moving microscopy stage, combined with the Wormlab software is a suitable setting to monitor the nicotine-dependent behavioral patterns in *C. elegans*.

The hardware settings for behavioral study were established according to the Worm Tracker system developed by the Schafer and Sternberg laboratory (MRC LMB, http://www.mrc-lmb.cam.ac.uk/wormtracker/index.php?action=hardware). We used the WormLab software to analyze videos taken by the Worm Tracker in this study. The WormLab is a multi-worm tracking and analysis software; it tracks the movements of worms in the view of observation, eliminating the need for a moving stage of the microscope (Figure 1). However, choosing of the worm populations scattered on different locations on tracking plates, needs to be done manually. For tracking, we observed the software performed better with 2–8 worms within the view. The magnification used is not as high as the Worm Tracker that tracks the movement of a single worm at a time, which is useful for delicate behavior observations. Another program known as the Parallel Worm Tracker, developed by the Goodman lab (Stanford University, Stanford, CA), is useful for simultaneously tracking a higher number of worms. However, the magnification of the Parallel Worm Tracker is lower than that of WormLab. The WormLab combines the advantageous features of both the Worm Tracker and the Parallel Worm Tracker. In this study we successfully defined the nicotine-dependent locomotion behavior indicating stimulation, adaption, and withdrawal responses using the speed parameter from the WormLab software. Our data indicated that the combined system is suitable in defining nicotine-associated locomotion behavior based on a relatively large worm population. The assay is more convenient and less time-consuming with relatively short video lengths (~ 5 minutes).

In the current study, the locomotion speed of naïve worms in nicotine-containing environments was significantly higher than in nicotine-free environments suggesting a stimulation/excitatory effect of nicotine on worms. The same effect was observed in other studies that observed a stimulation of locomotion in naïve worms when plated in nicotine containing environments (Feng *et al.*, 2006; Sobkowiak *et al.*, 2011). It is worth noting the data collection in Feng *et al's* study occurred during the first 2–16 minutes of exposure to the tracking plates. In this study a 30-minute acclimation period was used after transferring. We observed significantly more naïve worms who exhibited an abnormally high locomotion speed in nicotine-containing as compared to nicotine-free environments. This indicates that the stimulation effects of nicotine were still significant after 30 minutes of nicotine exposure. This finding is also in agreement with findings in Sobkowiak *et al*'s study, in which enhanced locomotion speed were observed during 0–300 min of naïve worms'

exposure to nicotine. In rodents, overall activity-enhancing effects was also observed in chronic nicotine treatment and in cessation (Faraday *et al.*, 2001).

Significant locomotion stimulation was observed during nicotine cessation in both nicotine treatment groups; this is in agreement with previous findings (Feng et al., 2006). Also stronger withdrawal stimulation on locomotion was observed in the higher nicotine treatment group (61.7 μM) as compared to the low group (6.17 μM). Although withdrawal stimulation is one aspect of nicotine-dependent behaviors, a return to baseline for withdrawal behaviors is another important aspect to demonstrate physical dependence, which warrants future investigations. In addition, the speed of 640 μm/sec displayed in nicotine-treated worms when they were transferred to a nicotine-free environment was much higher than naïve worms in a nicotine-containing environment (194 μm/sec). This suggests the withdrawal locomotion stimulation in nicotine-treated worms was much stronger than the acute-stimulation of naïve worms. The withdrawal locomotion stimulation effects may mimic withdrawal/craving activities in humans when tobacco use was abruptly stopped in smoking addicts (Henningfield *et al.*, 2009).

Nicotine-treated worms displayed significantly lower speeds (51.9 μm/sec for 6.17 μM group and 92.9 μm/sec for 61.7 μM group) on nicotine-containing plates as compared to naïve worms in the same concentration of nicotine-tracking plates (194 μm/sec); i.e. the same nicotine concentration did not induce the same stimulation effects in nicotine-treated worms. The observation that nicotine-treated worms in a nicotine-containing environment behaved similarly to the control group (naïve subjects) in a nicotine-free environment and different from the acute exposure group suggests nicotine-treated worms developed tolerance to the locomotor effects of nicotine. Indeed, the comparisons of all three conditions (naïve subjects on no-nicotine plates, naïve subjects on nicotine plates, nicotine-exposed subjects on nicotine plates) support the notion that tolerance to the locomotor effects of nicotine were observed. For the 61.7 μM exposure group, since the nicotine tracking plates only contain 6 μM of nicotine, considerably lower than the exposure level of 61.7 μM, the results indicate that a small amount of nicotine will greatly reduce withdrawal symptoms (i.e. locomotion speed reduced from 640 to 92.9 μm/sec). This observation, consistent with findings in other animal models, supports current nicotine replacement therapy using the low-dose nicotine to aid smoking cessation (Slawecki & Ehlers, 2002; Henningfield *et al.*, 2009).

In humans, blood nicotine concentration peaks at 0.5 μM after one cigarette consumption, (Pidoplichko *et al.*, 1997). In 2002, male smokers in the U.S. smoked on average 14.2 cigarettes per day and women smokers smoked 12.1 per day http://www.samhsa.gov/data/2k3/cigs/cigs.htm). The two concentrations used in current study were 6.17 and 61.7 μM in dosing medium. Given the *C. elegans*' cuticle is a significant barrier for drug permeability, the internal concentrations in worms' body fluid is likely to be substantially lower than the dosing medium concentration (Wolf & Heberlein, 2003). In this current study, the exposure starts from L4 stage and last for 24hr, 1/3 of *C. elegans* life cycle. For roughly translating to mammalian exposure, it may represent the period of adolescence to early adulthood. In other animal models, the adolescence period has been demonstrated to be

a vulnerable developmental stage for nicotine-induced rewarding activities (Slotkin, 2002; Belluzzi *et al.*, 2004).

At least 28 nAChR have been identified in *C. elegans* that are homologs of mammalian subunits (Jones & Sattelle, 2003; Rand, 2007). The ACR-16-like group contains 10 gene members: *acr-7, acr-9, acr-10, acr-11, acr-14, acr-15, acr-16, acr-19, acr-21*, and *acr-25*. The UNC-29-like group contains 4 members: *lev-1, acr-2, acr-3, unc-29* and the UNC-38-like group contains 3 members: *acr-6, unc-38*, and *unc-63*. There are 8 members in the DEG-3-like group: *des-2, deg-3, acr-5, acr-17, acr-18, acr-20, acr-23, acr-24* and the ACR-8-like group contains 3 members: *acr-8, lev-8*, and *acr-12*. Although functions of some nAChRs have been investigated, the *in vivo* expression pattern of these receptors has not yet been investigated in the context of nicotine-dependence. Studying *in vivo* expression of nicotine-induced nAChRs is beneficial to identify novel nicotine targets that are usually deemphasized by functional studies using mutants. Furthermore, the *in vivo* expression pattern is useful for identifying the combined effects of a group of genes, rather than only effects of a single or a few genes. Eleven genes were specifically up-regulated at the 61.7 μM nicotine treatment, in which worms showed significant nicotine-withdrawal behavior. Among those genes, five belong to the nicotine sensitive ACR-16 class (*acr-7*, *acr-11, acr-14, acr-16,* and *acr-21*). Although ACR-16 forms a homomeric nAChR in the *Xenopus* oocytes, our study showed a general up-regulation of other ACR-16-like genes, suggesting the likelihood of forming heteromeric receptors *in vivo* (Ballivet et al., 1996; Mongan et al., 2002). In vertebrates, the most abundant nAChR subunit expressed is the α7 subunit (Mineur & Picciotto, 2008). In *C. elegans*, the ACR-16-like group is the largest group containing the most α7-like nAChR subunit genes (*acr-7, acr-10, acr-16*, and *acr-21*) (Mongan *et al.*, 2002; Sattelle *et al.*, 2002). Our results showed that three out of four ACR-16 genes that code for α7 subunits were significantly up-regulated at the 61.7 μM group. Thereby these three genes (*acr-7*, *acr-16*, and *acr-21*) may be potential regulators of nicotine-dependent behaviors such as stimulation and withdrawal. The results also suggest that modulations of nAChRs were not specific to individual subtypes: two members (*acr-5* and *acr-17*) in the DEG-3-like group, five members (*acr-7, acr-11, acr-14, acr-16*, and *acr-21*) in ACR-16-like, two members (*acr-6* and *unc-63*) in the UNC-38-like group, four members (*lev-1, acr-2, acr-3*, and *unc-29*) in the UNC-29-like group, and one member in ACR-8-like group (*lev*-8) were specifically activated in the 61.7 μM treatment. Likewise, *ric-3*, which enhances the trafficking and assembly of nAChRs, was significantly up-regulated at both concentrations of nicotine. Under high concentrations, up-regulation of important nAChR genes coupled with the up-regulation of *ric-3*, may explain the increased sensitivity to nicotine and the resulting stronger nicotine-withdrawal locomotion stimulations observed in the 61.7 μM group. In addition, *unc-63* was significantly down regulated at both treatment groups. One study has demonstrated that *unc-63* down-regulation led to enhanced acquisition of tolerance (Matta *et al.*, 2007).

Chronic drug exposure usually results in desensitization of receptors. It was demonstrated that chronic exposure (16-hour) to nicotine (30 mM) resulted in insensitivity to the egg-laying stimulation effects of nicotine, suggesting the decreased nAChR activity/abundance following prolonged treatments (Waggoner *et al.*, 2000). This current study suggests that an

important determinant for nicotine tolerance and withdrawal would be the dosage. At a dosage of 30 mM chronic exposure, the *unc-29* was down-regulated and contributed to the loss of sensitivity to nicotinic agonists. In contrast, impacts of chronic (24-hour) exposure to relatively low levels of nicotine (6.17 and 61.7 μM) led to up-regulation of *unc-29* in this study. Interestingly, the nAChR up-regulation was also observed in humans following prolonged tobacco use (chronic exposure). The up-regulation in abstinent smokers persisted for at least 14 days after smoking cessation (Wonnacott, 1990). Our results suggested again that *C. elegans* mimic human reactions to nicotine at both molecular and behavioral levels.

In summary, the combined system (WormTracker and WormLab) is a suitable platform for defining nicotine-dependent phenotypic behaviors in *C. elegans*. The findings on *in vivo* expressions of nAChRs at mRNA level provide some useful information linking the genetic regulation with nicotine-dependent behaviors. However, more studies will be needed to establish causal relationships between individual and combined nAChR expressions with behavioral phenotypes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

Wormatlas. Wormbook; http://www.wormatlas.org/hermaphrodite/introduction/mainframe.htmhttp://www.wormbook.org/

Anderson GL, Boyd WA, Williams PL. Assessment of sublethal endpoints for toxicity testing with the nematode Caenorhabditis elegans. Environmental toxicology and chemistry/SETAC. 2001; 20:833–838. [PubMed: 11345460]

Anderson GL, Cole RD, Williams PL. Assessing behavioral toxicity with Caenorhabditis elegans. Environmental toxicology and chemistry/SETAC. 2004; 23:1235–1240. [PubMed: 15180374]

Azam L, Winzer-Serhan U, Leslie FM. Co-expression of alpha7 and beta2 nicotinic acetylcholine receptor subunit mRNAs within rat brain cholinergic neurons. Neuroscience. 2003; 119:965–977. [PubMed: 12831856]

Ballivet M, Alliod C, Bertrand S, Bertrand D. Nicotinic acetylcholine receptors in the nematode Caenorhabditis elegans. Journal of molecular biology. 1996; 258:261–269. [PubMed: 8627624]

Belluzzi JD, Lee AG, Oliff HS, Leslie FM. Age-dependent effects of nicotine on locomotor activity and conditioned place preference in rats. Psychopharmacology. 2004; 174:389–395. [PubMed: 14740150]

Benowitz NL. Drug therapy: Pharmacologic aspects of cigarette smoking and nicotine addition. New England Journal of Medicine. 1988; 319:1318–1330. [PubMed: 3054551]

Brenner S. The genetics of *Caenorhabidits elegans*. Genetics. 1974; 77:71–94. [PubMed: 4366476]

Cahir E, Pillidge K, Drago J, Lawrence AJ. The Necessity of alpha 4* Nicotinic Receptors in Nicotine-Driven Behaviors: Dissociation Between Reinforcing and Motor Effects of Nicotine. Neuropsychopharmacology. 2011; 36:1505–1517. [PubMed: 21430644]

Castillo M, Mulet J, Gutierrez LM, Ortiz JA, Castelan F, Gerber S, Sala S, Sala F, Criado M. Dual role of the RIC-3 protein in trafficking of serotonin and nicotinic acetylcholine receptors. J Biol Chem. 2005; 280:27062–27068. [PubMed: 15927954]

Changeux JP, Edelstein SJ. Allosteric receptors after 30 years. Neuron. 1998; 21:959–980. [PubMed: 9856454]

Culetto E, Baylis HA, Richmond JE, Jones AK, Fleming JT, Squire MD, Lewis JA, Sattelle DB. The Caenorhabditis elegans unc-63 gene encodes a Levamisole-sensitive nicotinic acetylcholine receptor alpha subunit. J Biol Chem. 2004; 279:42476–42483. [PubMed: 15280391]

Cutter AD, Dey A, Murray RL. Evolution of the Caenorhabditis elegans genome. Molecular biology and evolution. 2009; 26:1199–1234. [PubMed: 19289596]

Faraday MM, Elliott BM, Grunberg NE. Adult vs. adolescent rats differ in biobehavioral responses to chronic nicotine administration. Pharmacology Biochemistry and Behavior. 2001; 70:475–489.

Feng Z, Li W, Ward A, Piggott BJ, Larkspur ER, Sternberg PW, Xu XZ. A C. elegans model of nicotine-dependent behavior: regulation by TRP-family channels. Cell. 2006; 127:621–633. [PubMed: 17081982]

Francis MM, Evans SP, Jensen M, Madsen DM, Mancuso J, Norman KR, Maricq AV. The Ror receptor tyrosine kinase CAM-1 is required for ACR-16-mediated synaptic transmission at the C. elegans neuromuscular junction. Neuron. 2005; 46:581–594. [PubMed: 15944127]

Gerzanich V, Wang F, Kuryatov A, Lindstrom J. alpha 5 Subunit alters desensitization, pharmacology, Ca++ permeability and Ca++ modulation of human neuronal alpha 3 nicotinic receptors. The Journal of pharmacology and experimental therapeutics. 1998; 286:311–320. [PubMed: 9655874]

Gottschalk A, Almedom RB, Schedletzky T, Anderson SD, Yates JR, Schafer WR. Identification and characterization of novel nicotinic receptor-associated proteins in Caenorhabditis elegans. Embo J. 2005; 24:2566–2578. [PubMed: 15990870]

Halevi S, McKay J, Palfreyman M, Yassin L, Eshel M, Jorgensen E, Treinin M. The C. elegans ric-3 gene is required for maturation of nicotinic acetylcholine receptors. Embo J. 2002; 21:1012–1020. [PubMed: 11867529]

Henningfield JE, Shiffman S, Ferguson SG, Gritz ER. Tobacco dependence and withdrawal: Science base, challenges and opportunities for pharmacotherapy. Pharmacology & Therapeutics. 2009; 123:1–16. [PubMed: 19362108]

Hernando G, Berge I, Rayes D, Bouzat C. Contribution of Subunits to Caenorhabditis elegans Levamisole-Sensitive Nicotinic Receptor Function. Mol Pharmacol. 2012; 82:550–560. [PubMed: 22734069]

Jones AK, Davis P, Hodgkin J, Sattelle DB. The nicotinic acetylcholine receptor gene family of the nematode Caenorhabditis elegans: an update on nomenclature. Invertebrate neuroscience: IN. 2007; 7:129–131. [PubMed: 17503100]

Jones AK, Sattelle DB. Functional genomics of the nicotinic acetylcholine receptor gene family of the nematode, Caenorhabditis elegans. Bioessays. 2003; 26:39–49. [PubMed: 14696039]

Jospin M, Qi YB, Stawicki TM, Boulin T, Schuske KR, Horvitz HR, Bessereau JL, Jorgensen EM, Jin YS. A Neuronal Acetylcholine Receptor Regulates the Balance of Muscle Excitation and Inhibition in Caenorhabditis elegans. Plos Biol. 2009:7.

Karlin A. Emerging structure of the nicotinic acetylcholine receptors. Nature reviews Neuroscience. 2002; 3:102–114.

Lansdell SJ, Gee VJ, Harkness PC, Doward AI, Baker ER, Gibb AJ, Millar NS. RIC-3 enhances functional expression of multiple nicotinic acetylcholine receptor subtypes in mammalian cells. Mol Pharmacol. 2005; 68:1431–1438. [PubMed: 16120769]

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. Methods. 2001; 25:402–408. [PubMed: 11846609]

Marks MJ. Genetic matters: Thirty years of progress using mouse models in nicotinic research. Biochemical Pharmacology. 2013; 86:1105–1113. [PubMed: 23747348]

Matta SG, Balfour DJ, Benowitz NL, Boyd RT, Buccafusco JJ, Caggiula AR, Craig CR, Collins AC, Damaj MI, Donny EC, Gardiner PS, Grady SR, Heberlein U, Leonard SS, Levin ED, Lukas RJ, Markou A, Marks MJ, McCallum SE, Parameswaran N, Perkins KA, Picciotto MR, Quik M, Rose JE, Rothenfluh A, Schafer WR, Stolerman IP, Tyndale RF, Wehner JM, Zirger JM. Guidelines on

nicotine dose selection for in vivo research. Psychopharmacology. 2007; 190:269–319. [PubMed: 16896961]

Millar NS. RIC-3: a nicotinic acetylcholine receptor chaperone. British journal of pharmacology. 2008; 153(Suppl 1):S177–183. [PubMed: 18246096]

Mineur YS, Picciotto MR. Genetics of nicotinic acetylcholine receptors: Relevance to nicotine addiction. Biochemical pharmacology. 2008; 75:323–333. [PubMed: 17632086]

Mongan NP, Baylis HA, Adcock C, Smith GR, Sansom MS, Sattelle DB. An extensive and diverse gene family of nicotinic acetylcholine receptor alpha subunits in Caenorhabditis elegans. Receptors Channels. 1998; 6:213–228. [PubMed: 10100329]

Mongan NP, Jones AK, Smith GR, Sansom MSP, Sattelle DB. Novel alpha 7-like nicotinic acetylcholine receptor subunits in the nematode Caenorhabditis elegans. Protein Sci. 2002; 11:1162–1171. [PubMed: 11967372]

Petrash HA, Philbrook A, Haburcak M, Barbagallo B, Francis MM. ACR-12 Ionotropic Acetylcholine Receptor Complexes Regulate Inhibitory Motor Neuron Activity in Caenorhabditis elegans. Journal of Neuroscience. 2013; 33:5524–5532. [PubMed: 23536067]

Pidoplichko VI, DeBiasi M, Williams JT, Dani JA. Nicotine activates and desensitizes midbrain dopamine neurons. Nature. 1997; 390:401–404. [PubMed: 9389479]

Ramot D, Johnson BE, Berry TL, Carnell L, Goodman MB. The Parallel Worm Tracker: A Platform for Measuring Average Speed and Drug-Induced Paralysis in Nematodes. Plos One. 2008:3.

Rand JB. Acetylcholine. WormBook: the online review of C. elegans biology. 2007:1–21. [PubMed: 18050502]

Riddle, DL.; Blumenthal, T.; Meyer, BJ.; Priess, JR. Introduction to C. elegans. In: Riddle, DL.; Blumenthal, T.; Meyer, BJ.; Priess, JR., editors. C. elegans II. Cold Spring Harbor (NY): 1997.

Roh JYPY, Choi J. A cadmium toxicity assay using stress responsive *Caenorhabditis elgans* mutant strains. Environmental Toxicology and Pharmacology. 2009; 28:409–413. [PubMed: 21784035]

Rose JK, Miller MK, Crane SA, Hope KA, Pittman PG. Parental and larval exposure to nicotine modulate spontaneous activity as well as cholinergic and GABA receptor expression in adult C. elegans. Neurotoxicology and teratology. 2013; 39:122–127. [PubMed: 23906944]

Sattelle DB, Culetto E, Grauso M, Raymond V, Franks CJ, Towers P. Functional genomics of ionotropic acetylcholine receptors in Caenorhabditis elegans and Drosophila melanogaster. Novart Fdn Symp. 2002; 245:240–260.

Saur T, DeMarco SE, Ortiz A, Sliwoski GR, Hao LM, Wang X, Cohen BM, Buttner EA. A Genome-Wide RNAi Screen in Caenorhabditis elegans Identifies the Nicotinic Acetylcholine Receptor Subunit ACR-7 as an Antipsychotic Drug Target. Plos Genet. 2013:9.

Schafer WR. Addiction research in a simple animal model: the nematode Caenorhabditis elegans. Neuropharmacology. 2004; 47(Suppl 1):123–131. [PubMed: 15464131]

Sellings L, Pereira S, Qian C, Dixon-McDougall T, Nowak C, Zhao B, Tyndale RF, van der Kooy D. Nicotine-motivated behavior in Caenorhabditis elegans requires the nicotinic acetylcholine receptor subunits acr-5 and acr-15. Eur J Neurosci. 2013; 37:743–756. [PubMed: 23351035]

Sherlekar AL, Janssen A, Siehr MS, Koo PK, Caflisch L, Boggess M, Lints R. The C. elegans male exercises directional control during mating through cholinergic regulation of sex-shared command interneurons. PloS one. 2013; 8:e60597–e60597. [PubMed: 23577128]

Slawecki CJ, Ehlers CL. Lasting effects of adolescent nicotine exposure on the electroencephalogram, event related potentials, and locomotor activity in the rat. Developmental Brain Research. 2002; 138:15–25. [PubMed: 12234654]

Slotkin TA. Nicotine and the adolescent brain: Insights from an animal model. Neurotoxicology and Teratology. 2002; 24:369–384. [PubMed: 12009492]

Smith MA Jr, Zhang Y, Polli JR, Wu H, Zhang B, Xiao P, Farwell MA, Pan X. Impacts of chronic low-level nicotine exposure on Caenorhabditis elegans reproduction: Identification of novel gene targets. Reproductive Toxicology. 2013; 40:69–75. [PubMed: 23735997]

Sobkowiak R, Kowalski M, Lesicki A. Concentration- and time-dependent behavioral changes in Caenorhabditis elegans after exposure to nicotine. Pharmacology, biochemistry, and behavior. 2011; 99:365–370.

Taki FA, Zhang B. Determination of reliable reference genes for multi-generational gene expression analysis on C. elegans exposed to abused drug nicotine. Psychopharmacology. 2013; 230:77–88. [PubMed: 23681163]

Touroutine D, Fox RM, Von Stetina SE, Burdina A, Miller DM, Richmond JE. acr-16 encodes an essential subunit of the levamisole-resistant nicotinic receptor at the Caenorhabditis elegans neuromuscular junction. J Biol Chem. 2005; 280:27013–27021. [PubMed: 15917232]

Towers PR, Edwards B, Richmond JE, Sattelle DB. The Caenorhabditis elegans lev-8 gene encodes a novel type of nicotinic acetylcholine receptor alpha subunit. Journal of Neurochemistry. 2005; 93:1–9. [PubMed: 15773900]

Treinin M, Gillo B, Liebman L, Chalfie M. Two functionally dependent acetylcholine subunits are encoded in a single Caenorhabditis elegans operon. P Natl Acad Sci USA. 1998; 95:15492–15495.

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. The Journal of neuroscience: the official journal of the Society for Neuroscience. 2000; 20:8802–8811. [PubMed: 11102488]

Wolf FW, Heberlein U. Invertebrate Models of Drug Abuse. Journal of Neurobiology. 2003; 54:161–178. [PubMed: 12486703]

Wonnacott S. The paradox of nicotinic acetylcholine receptor upregulation by nicotine. Trends in pharmacological sciences. 1990; 11:216–219. [PubMed: 2200178]

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]

Highlights

- Wormtracker works with Wormlab for characterization of nicotine-dependent behaviors.
- Chronic nicotine treatment induced withdrawal stimulation of locomotion behaviors.
- nAChR genes were disregulated in *C. elegans* displaying nicotine-dependent behaviors.

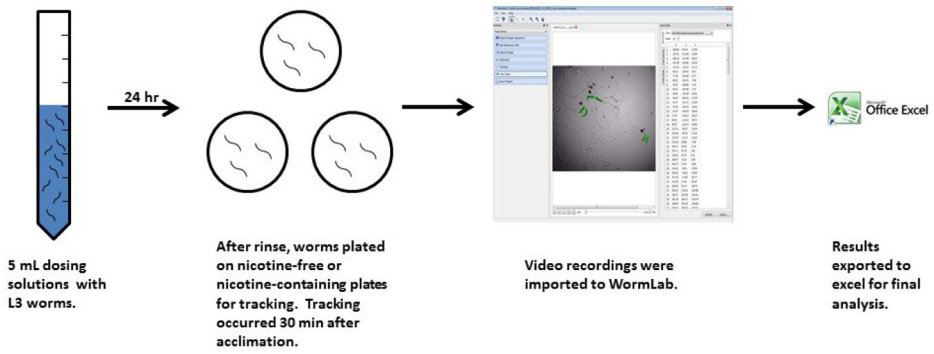

Figure 1.

L3 worms were treated in K-media liquid solutions for 24 hours and then transferred to 3 cm tracking plates containing or without nicotine. After 30 minutes of acclimation, videos (5 minutes) were recorded and imported into the WormLab analytical software where the results were then exported to excel for further analysis.

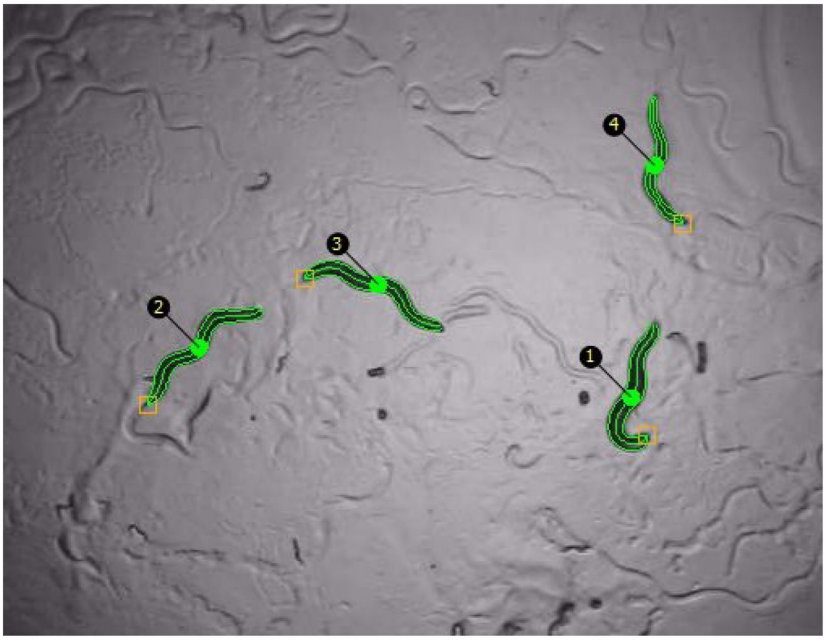

Figure 2. Field of view in WormLab

After importing the tracking videos into WormLab, worms were selected and highlighted for tracking by the program. Each worm was labeled with a numerical value and the orange box indicated the head of worm. Worm locomotion speeds were then exported from WormLab into a Microsoft Excel file.

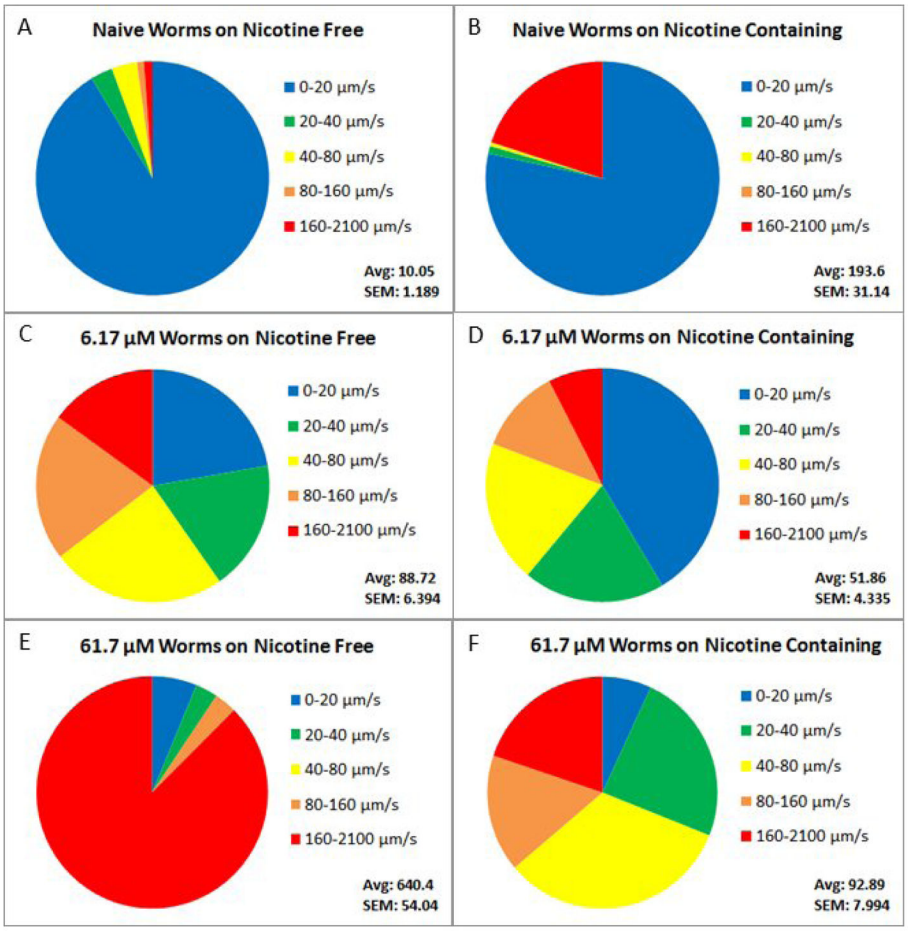

Figure 3. The distribution of worm polulations into different speed ranges on nicotine-free and nicotine-containing (6 µM) tracking plates

All worms were placed on tracking plates for a 30-minute acclimation period and then video-recorded for 5 minutes. Naïve worms on A) nicotine-free and B) Nicotine-containing plates; worms exposed to 6.17 µM nicotine for 24 hours were then recorded on C) nicotine-free and D) nicotine-containing plates; and worms exposed to 61.7 µM nicotine for 24 hours were then recorded on E) nicotine-free and F) nicotine-containing plates.

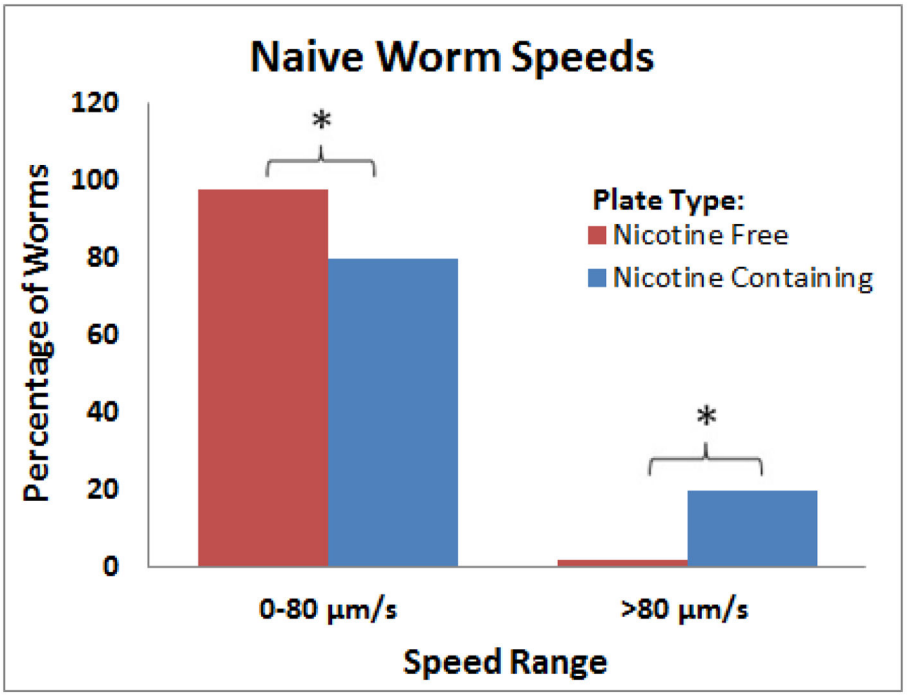

Figure 4. Acute nicotine treatment induced locomotion stimulation behaviors in naïve worms

The locomotion speeds of naïve worms on nicotine-free (n= 423 worms in total) and nicotine-containing plates (n =190 worms in total). Six independent experiments were performed. All worms were placed on tracking plates for 30 minutes to acclimate and then video-recorded for 5 minutes. A majority of naïve worms (98%) plated on nicointe-free plates were in the speed range of 0–80 µm/s. A significant portion of the worm population (20%) exhibited abnormally high speeds of > 80 µm/s on nicotine-containing plates. Chi-square analysis revealed statistically significante differences in naïve worm population distributions between nicotine-free and nicotine-containing environments (*: $p < 0.05$).

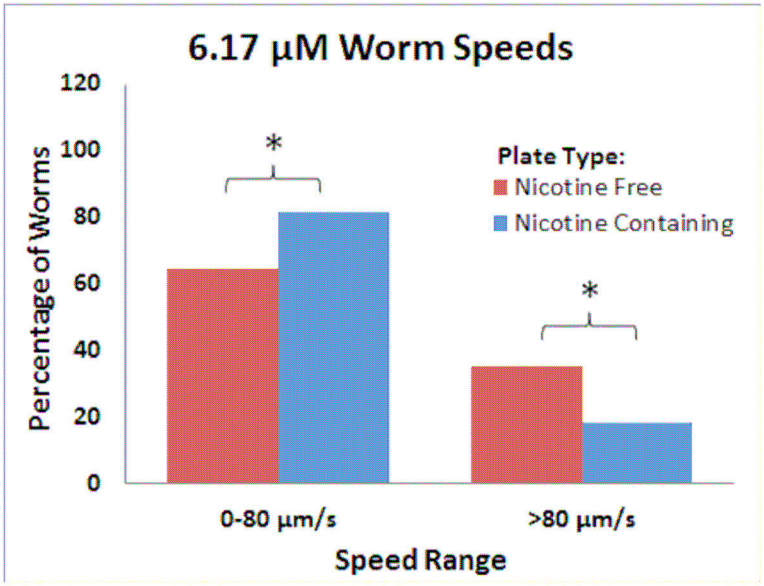

Figure 5. Chronic nicotine treatment (6.17 µM for 24 hours) induced locomotion behaviors

The locomotion speeds of nicotine treated worms on nicotine-free (n= 387 worms in total) and nicotine-containing plates (n =251 worms in total). Six independent experiments were performed. All worms were placed on tracking plates for 30 minutes to acclimate and then video-recorded for 5 minutes. A significant portion of the worm population (35%) exhibited abnormally high speeds of > 80 µm/s on nicotine-free plates. Chi-square analysis revealed statistically significant differences in worm population distributions between nicotine-free and nicotine-containing environments (*: $p < 0.05$).

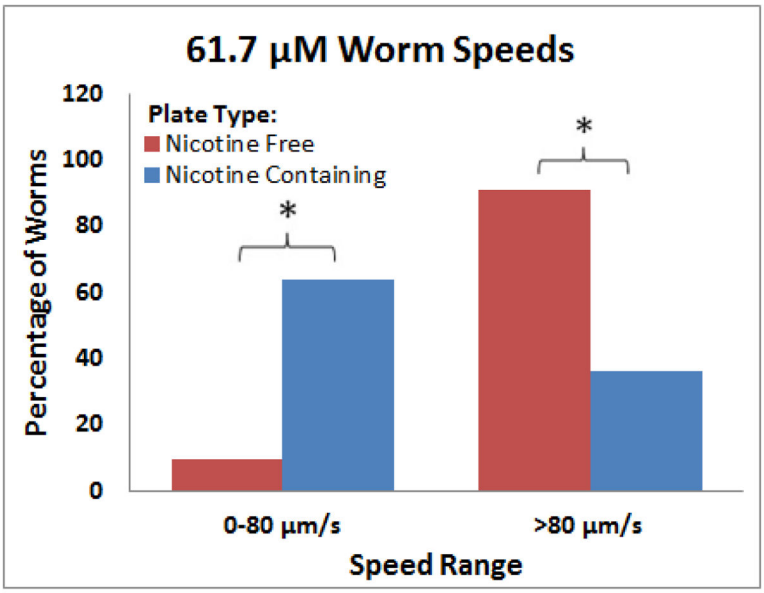

Figure 6. Chronic nicotine treatment (61.7 µM for 24 hours) induced locomotion behaviors

The locomotion speeds of nicotine treated worms on nicotine-free on nicotine-free (n= 64 worms in total) and nicotine-containing plates (n = 116 worms in total). Six independent experiments were performed. All worms were placed on tracking plates for 30 minutes to acclimate and then video-recorded for 5 minutes. A significant portion of the worm population (91%) exhibited abnormally high speeds of > 80 µm/s on nicotine-free plates. Chi-square analysis revealed statistically significant differences in worm population distributions between nicotine-free and nicotine-containing environments (*: $p < 0.05$).

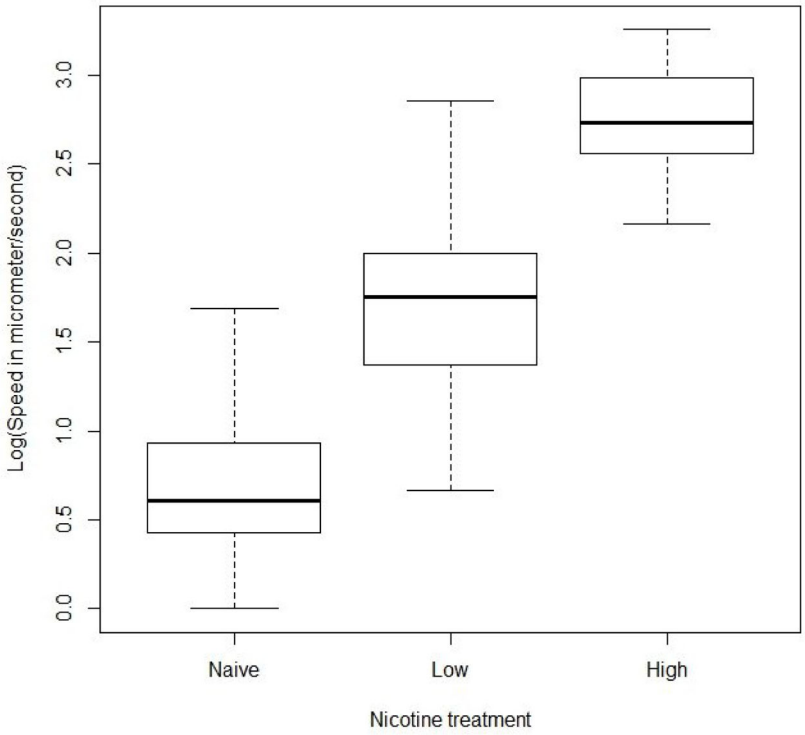

Figure 7. The relationship between nicotine dosage and locomotion speed during nicotine cessation

The X-axis are different groups of nicotine-treated worms, naïve worms, low (6.17 μM), and high (61.7 μM) nicotine treatments. The Y-axis uses logarithmic scale with 10 as bases (Log_{10} (speed value in μm/sec). The solid lines within the boxes indicate median speed values and the lower and upper hinges indicate data within 25 and 75 percentiles. The whiskers show the largest/smallest speed values that fall within a distance of 1.5 times IQR (interquartile range) from the upper and lower hinges. The two groups of nicotine-treated worms have higher locomotion speeds compared to naïve worms when on nicotine-free environment ($p<0.001$, ANOVA). Also the high nicotine treated group display significantly higher locomotion speed when on nicotine-free plates compared to low nicotine-treated group ($p<0.001$, ANOVA).

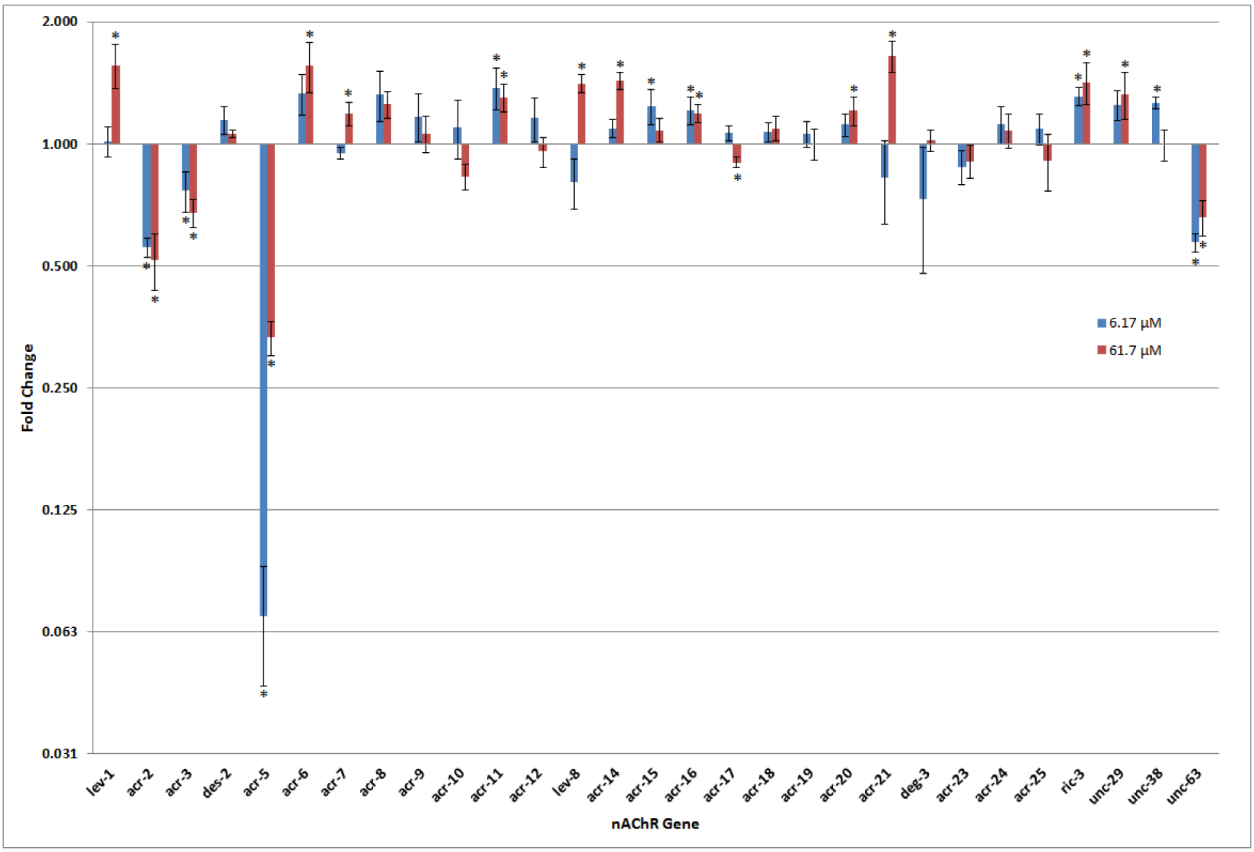

Figure 8. The expression fold changes of the 28 nAChR genes and *ric*-3 in *C. elegans* after being exposed to nicotine for 24 hours

All Ct values were normalized using F35G12.2 mRNA then compared to a control value of 1 (> 1 means up-regulated, < 1means down-regulated). *Y-axis* uses a logarithmic scale with a base of 2 ($\log_2$). The error bars represent the standard error of fold change for four biological replicates per gene treatment (n=4). Results were deemed significant if $p < 0.05$ (*).

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Table 1

The descriptions and functions of the 28 nAChR genes within the *C. elegans* genome.

Gene Symbol	Locus Tag	Gene Description	Subunit Type	Muscle & Neurons	Formation of Channels	Lev-sensitive	Signal Transduction	Ref
acr-2	K11G12.2	AcetylCholine Receptor	**non-α**	✓	✓			(Mongan *et al.*, 2002; Jospin *et al.*, 2009)
acr-3	K11G12.7	AcetylCholine Receptor	**non-α**	✓	✓			(Mongan *et al.*, 2002)
acr-5	K03F8.2	AcetylCholine Receptor	**α**	✓				(Mongan *et al.*, 1998; Sellings *et al.*, 2013)
acr-6	ZK973.5	AcetylCholine Receptor	**α**	✓			✓	(Mongan *et al.*, 1998; Mongan *et al.*, 2002)
acr-7	T09A5.3	AcetylCholine Receptor	**α**	✓	✓		✓	(Mongan *et al.*, 1998; Sattelle *et al.*, 2002; Saur *et al.*, 2013)
acr-8	ZC504.2	AcetylCholine Receptor	**α**	✓		✓		(Hernando *et al.*, 2012; Zhang *et al.*, 2012)
acr-9	C40C9.2	AcetylCholine Receptor	**non-α**	✓				(Mongan *et al.*, 1998)
acr-10	R02E12.8	AcetylCholine Receptor	**α**	✓	✓		✓	(Mongan *et al.*, 1998; Sattelle *et al.*, 2002; Jones & Sattelle, 2003)
acr-11	D2092.3	AcetylCholine Receptor	**α**	✓			✓	(Mongan *et al.*, 2002; Jones & Sattelle, 2003)
acr-12	R01E6.4	AcetylCholine Receptor	**α**	✓			✓	(Gottschalk *et al.*, 2005; Petrash *et al.*, 2013)
acr-14	T05C12.2	AcetylCholine Receptor	**non-α**	✓				(Jones & Sattelle, 2003)
acr-15	F25G6.4	AcetylCholine Receptor	**α**	✓			✓	(Mongan *et al.*, 1998; Sellings *et al.*, 2013)
acr-16	F25G6.3	AcetylCholine Receptor	**α**	✓	✓		✓	(Mongan *et al.*, 2002; Jones & Sattelle, 2003; Francis *et al.*, 2005; Touroutine *et al.*, 2005)
acr-17	F53E10.2	AcetylCholine Receptor	**α**	✓				(Mongan *et al.*, 2002)
acr-18	F28F8.1	AcetylCholine Receptor	**α**	✓			✓	(Jones & Sattelle, 2003; Sherlekar *et al.*, 2013)
acr-19	C31H5.3	AcetylCholine Receptor	**α**	✓				(Mongan *et al.*, 2002)
acr-20	R06A4.10	AcetylCholine Receptor	**α**	✓				(Jones & Sattelle, 2003)
acr-21	F27B3.2	AcetylCholine Receptor	**α**	✓				(Mongan *et al.*, 2002)

Gene Symbol	Locus Tag	Gene Description	Subunit Type	Muscle & Neurons	Formation of Channels	Lev-sensitive	Signal Transduction	Ref
acr-23	F59B1.9	AcetylCholine Receptor	**α**	✓				(Jones & Sattelle, 2003)
acr-24	Y73F8A.30	AcetylCholine Receptor	**α**	✓	✓			(Jones & Sattelle, 2003; Jones *et al.*, 2007)
acr-25	Y73B6BL.42	AcetylCholine Receptor	**α**	✓	✓			(Jones & Sattelle, 2003; Jones *et al.*, 2007)
deg-3	K03B8.9	DEGeneration of certain neurons	**α**	✓	✓			(Treinin *et al.*, 1998; Jones & Sattelle, 2003)
des-2	T26H10.1	DEgeneration Suppressor	**α**	✓	✓			(Treinin *et al.*, 1998; Jones & Sattelle, 2003)
lev-1	F09E8.7	LEVamisole resistant	**non-α**	✓	✓	✓		(Mongan *et al.*, 2002; Jones & Sattelle, 2003; Culetto *et al.*, 2004)
lev-8	C35C5.5	LEVamisole resistant	**α**	✓		✓		(Mongan *et al.*, 2002; Towers *et al.*, 2005)
unc-29	T08G11.5	UNCoordinated	**non-α**	✓	✓	✓		(Mongan *et al.*, 2002; Jones & Sattelle, 2003; Culetto *et al.*, 2004)
unc-38	F21F3.5	UNCoordinated	**α**	✓	✓	✓		(Mongan *et al.*, 2002; Jones & Sattelle, 2003; Culetto *et al.*, 2004)
unc-63	Y110A7A.3	UNCoordinated	**α**	✓	✓	✓		(Jones & Sattelle, 2003; Culetto *et al.*, 2004)

Table 2

Primer information for the 29 tested genes.

Gene Symbol	Locus Tag	Forward Primer	Reverse Primer	Amplicon size
acr-2	K11G12.2	AGTGCACTGCAATGGTGGTCACG	ACTCTCGGAAGAGATCTATGACC	161
acr-3	K11G12.7	TGAAACGAGAGAAACGTGAGAG	TGCGTTGTGATATACTCGATCGC	184
acr-5	K03F8.2	ATGGACACAAGTCAGGCGAGAGG	TACAGCAAATGTGAATCCGCAG	132
acr-6	ZK973.5	ACTGGTTCATTGAAAAGCTCGG	TGGCTGATAATGCATTGACTTGC	158
acr-7	T09A5.3	AGCAGATCAATGTGTTCAGTGTGC	CAATGTTTCTGTAGGAAACGG	118
acr-8	ZC504.2	AACAGCAGAAGAAGATGATGGG	TAACCGTCCATTCAACTGACG	127
acr-9	C40C9.2	ACACCTTAAAGCAGAGAAAGCTG	AATCTATAGTCTGCTGTCTCC	152
acr-10	R02E12.8	TCTCGTCGCTCACCCTTATCAGC	TGTGAATATTCTGCACTTGACGG	90
acr-11	D2092.3	GGAAGTGTTGCGGAGACAAAGC	AACTGAGCCACGCATTCACTG	175
acr-12	R01E6.4	AAGACACTTCACATTCCAAGCG	ATCGGTCAAGATTGTGATGTCG	96
acr-14	T05C12.2	GATTTACTCTACCACCAGACGC	TGAAGACACATGAACAGGCTAC	180
acr-15	F25G6.4	CGTTCGAATATGCAGATGGACC	CTTCTGTAACACCCGCAATCTC	146
acr-16	F25G6.3	ACTAAGCCAAATCGTCACTCGG	TCATTGTACTTGGAATACTGCC	169
acr-17	F53E10.2	TGGATGTGTTGAGTTCTGTGC	TGTCGTAGAGCATGAAGGACG	168
acr-18	F28F8.1	ACAGAGCTTCTTCTTCACCAACG	TTCATTAGCGACGATGTCAAC	189
acr-19	C31H5.3	ATCATCGATCACCAGAACAG	TTCAAGAACATCAGGACGTTCC	107
acr-20	R06A4.10	AGGAAGGACGTTCAGACATCG	TCCAACAGAGACACATCCTGATC	194
acr-21	F27B3.2	TTGCTGTCCAGAACCATATCC	TGACAGAAGTGATGTGATGC	181
acr-23	F59B1.9	ATGTGTCGTCTTCAGTTCACG	AAGTGGAACACATGTAGACGTCG	131
acr-24	Y73F8A.30	AGAAGGACAGAAGAACTCGAAG	TTGGTGCTGCAAGTGGAGC	131
acr-25	Y73B6BL.42	TTCGTCTCCTGATGCTTG	TTCTCTGTTGATCCAACC	144
deg-3	K03B8.9	AAGGAAGTGGACGAGATGC	TGTCACGATGACGACTGCTC	117
des-2	T26H10.1	TCAATTTCACCTTCAGCTCCACC	ATTGCGGCAAATGTGTTGTCC	167
lev-1	F09E8.7	TCTGTCAATTGTTGTGTTCCTGC	AACCCAAGGTGGTAATCGATGGG	187
lev-8	C35C5.5	TTACTGCATCTGTTACGCAACG	TGTTGGTTGTGGAAATGTGCAG	196
ric-3	T14A8.1	TGGGTCGTTCACCACCATCAAGT	GCACCACCACCTGGATGACGTT	103
unc-29	T08G11.5	CGGATGCCACAATGGGTTCGAG	ACGGACAGCGCTTCGCTCAG	99

Gene Symbol	Locus Tag	Forward Primer	Reverse Primer	Amplicon size
unc-38	F21F3.5	ACCTGGGAGCCACCAGCAATC	CAGGAGCCGAACTTCAAATGGCA	98
unc-63	Y110A7A.3	TGCGAACAGAGATGCGAATCGG	CGTCACCACGAGTGTCTCTCCG	100