Chemotaxis Response in *Caenorhabditis elegans* to an Olfactory Repellent Paired with Nicotine

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Recent studies in *C. elegans*, a model organism with molecular homologies to mammals, suggests *C. elegans* display a preference for environments associated with drugs of abuse after being exposed and conditioned with them. In addition, chronic exposure to these drugs can cause the worms to adopt altered behaviors as a result, such as showing preference for a chemical they are normally repelled from. Our study examined the influence of nicotine when paired with undiluted benzaldehyde, a known repellent, for differing amounts of time. Our results show a significant difference between non-conditioned and 60 minute conditioned groups. Further analysis shows a significant difference between number of worms at the test spot between the non-conditioned and the 15 and 30 minute conditioned groups as well as the 15 minute and 30 minute conditioned group. This shows the reward-type effect of nicotine on *C. elegans* behavior. Moreover, changes in behavior seem to show trends as early as 15 minutes of exposure. By studying addiction-related behaviors in *C. elegans*, it may be possible to understand and identify underlying mechanisms of addiction and nicotine motivational molecular pathways and eventually to develop new treatments against nicotine addiction.

INTRODUCTION

Smoking and tobacco are two of the leading causes of preventable death around the world. Use of such products often begins during adolescence and shows a co-morbid correlation with other addictions, such as alcohol abuse, making it an important topic of public health concern due to the addictive properties caused by the presence of nicotine, a highly addictive ingredient in cigarettes. Despite the numerous known health risks, tobacco use and smoking are increasing to include approximately one-third of the world population. In turn, tobacco use is responsible for over 400,000 deaths a year (Dani & Harris, 2005). While many people try to quit, it is extremely difficult due to nicotine. Nicotine is the leading psychoactive ingredient in cigarettes and produces a reward-type mechanism mostly by improving mood. This frequently leads to addiction, reinforced by further nicotine intake (Dani & Harris, 2005; Donny et al., 2007).

Currently, three main treatments are available to combat tobacco use, including nicotine replacement, bupropion and varenicline. While these treatments help more users quit compared to no treatment, only 20% quit long term (Sellings et al., 2013). Previous studies have been performed on rodents to understand the mechanisms in nicotine-motivated behavior. However, more recently *Caenorhabditis elegans* has been used to study nicotine effects due to their simpler, well-known, and detailed nervous systems, consisting of just 302 neurons that are easily identified in their transparent body. Nicotine-focused studies in *C. elegans* were comparable to those in the rodents and the worms have since become a model organism for such studies, especially due to their high degree of homology with mammals and their short generation time.
One site of homology between *C. elegans* and humans are nicotinic receptor subunits, specifically acetylcholine receptors, that play a role in behavior (Sellings et al., 2013). In the *C. elegans* nervous system, three particular sensory neuron pairs can detect odors. AWB neuron pairs are wired for repulsion while AWA and AWC neurons are wired for attraction (Bargmann & Horvitz, 1991; Bargmann et al., 1993; Troemel et al., 1997). Nicotine is likely detected by the neurons wired for attraction, AWA or AWC, but could also be detected through taste. However, these behavioral responses exhibit plasticity through associative learning, which involves changing a behavior to a conditioned stimulus by pairing it with an unconditioned stimulus, leading to adaptation, or a loss of attraction. (Levitan & Kaczmarek, 1991; Colbert & Bargmann, 1995; Torayama et al., 2007). Previous studies have displayed adaptation through the use of benzaldehyde and isoamyl alcohol when paired with starvation. (Pereira & van der Kooy, 2012; Nuttley et al. 2002).

Sellings et al. (2013) demonstrated reversal of starvation-induced conditioning away from butanone, also sensed by an AWC neuron, by pairing butanone with nicotine under the starving conditions. This is conditioning because typically when butanone is paired only with starvation, the worms show a reduced approach to butanone, but when also paired with nicotine, there is an increased approach. Based on these results, nicotine seems to act as a positive reward system. As stated above, mammals are homologous with *C. elegans* in certain nicotinic receptors. Sellings et al. also included acetylcholine, or acr, and dopamine, or dop, mutants in their study. The results showed that acr-5 and acr-15 mutants as well as dop-1 and dop-2 mutants reduced nicotine approach and did not exhibit the conditioned behavior. Through these studies, they hoped to identify genes involved in the mechanism that controls nicotine-motivated behavior (Sellings et al., 2013).

In the present study, I sought to examine the effects on the behavior and chemotactic response when nicotine is conditioned and paired with a natural repellent: undiluted benzaldehyde. This may be useful in identification of novel genes implicated in the control of nicotine-motivated behavior as well as developing drugs to combat nicotine addiction.

**MATERIALS AND METHODS**

**Organisms**
N2 Bristol wild-type worms and *E. coli* OP50, the food source, were obtained from the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, MN, USA). All worms were young adults and adults.

**LB agar**
LB agar consisted of 10 g Bacto-tryptone, 5 g Bacto-yeast, 5 g NaCl, and 15 g agar (all from Sigma), all dissolved in 1 L of water with a final pH of 7.5 and sterilized by autoclaving. The agar was poured into 10 cm petri dishes and incubated overnight at 37°C.

**L broth**
L broth consisted of 10 g Bacto-tryptone, 5 g Bacto-yeast, and 5 g NaCl, dissolved in 1 L of water with an adjusted pH of 7, using 1 M NaOH to adjust, and sterilized by autoclaving. LB
plates and L broths were stored at 4°C throughout experimentation and were fresh throughout the experiment.

**Nematode Growth Medium**
Nematode growth media (NGM) plates were made by mixing 3 g NaCl, 17 g agar, 2.5 g peptone, and 975 mL of water in a 2 L flask. After autoclaving, 1 mL of 1 M MgSO₄, 1 mL of 5 mg/mL cholesterol in ethanol, 1 mL 1 M CaCl₂, and 25 mL 1 M KPO₄ buffer (all sterile) were added before pouring into 10 cm diameter plates. They were stored at 4°C.

**OP50 Maintenance**
After incubation, a sterile 15 mL. conical tube of L broth was aseptically inoculated with one OP50 colony and incubated overnight at 37°C. Under sterile conditions, about half a milliliter of this OP50 culture was pipetted to the middle of the NGM plate and then spread using a “hockey stick” glass rod. The lawn was limited to the center area of the plate to prevent worms from crawling up the sides of the plate and drying out. These “seeded” plates were incubated overnight at 37°C before storing at 4°C for up to 3 weeks. The worm lineage was continued through the process of chunking: A sterilized scalpel was used to cut a 1 small square from a seeded plate and transferred upside-down onto a newly seeded plate for worms to spread on the new lawn. Chunking onto seeded NGM was done every 4-7 days. Worm growth plates were incubated at 21°C.

**Chemotaxis Plates**
Chemotaxis plates were made by autoclaving 1.6 g agar per 100 mL of deionized water. After autoclaving, the agar was kept in 50°C water bath until equilibrated. Then 500 µL potassium phosphate, 100 µL calcium chloride, and 100 µL magnesium sulfate (all sterile) were added. After combining, approximately 10 mL of agar was poured per circular 10 cm petri dish. Plates were allowed to cool at room temperature for 24 hours and stored at 4°C.

**Conditioning Procedure**
All undiluted benzaldehyde/nicotine pairing assays were based on and modified from Sellings’ butanone conditioning assay (Sellings et al., 2013.) Conditioning assays (N=9) were performed at room temperature on a 10-cm unseeded NGM plate. Worms were used four days after being plated on NGM plates seeded with *E. Coli* OP50 at 21°C. Ten µL 50 mM nicotine was plated in the center and diffused through the agar for 3 hours at room temperature and then the plate was sealed with Parafilm. After 3 hours, N2 worms were washed twice off a growth plate with S-basal buffer and once with deionized water, then 10 µL of worm pellet were placed on the nicotine spot. Four µL of undiluted benzaldehyde was placed on 6 pieces of Parafilm and placed on the lid and the inverted petri dish was sealed with Parafilm (Fig. 1.) After a set amount of time, worms were washed from the conditioning plate twice using deionized water. They were then tested for chemotaxis to undiluted benzaldehyde.

![Nicotine Spot](image1)

**Parafilm with bz**
FIG. 1. Conditioning Assay based on Sellings et al. butanone conditioning assay.

Chemotaxis Assay
Chemotaxis assay plates were performed with standard 10-cm circular plates. Control, test, and plating spots were marked on the plate. The control and test spots were marked 1.5 cm from the plate edge with a 2 cm diameter circle around it and the worm spot (origin) was marked 1 cm below the center of the plate, unless otherwise noted. Worms were washed twice with deionized water off the conditioning plate, unless otherwise noted. During the washes, 1 µL of 1 M sodium azide was added to the center of the control and test spots. Ten µL of worms were plated on the origin, then 1 µL of undiluted benzaldehyde was spotted at the test spot and 1 µL of deionized water was spotted at the control spot. The assays were left at room temperature for 60 minutes and the worms were counted in the control and test spots every 15 minutes with the lid on (Fig. 2.)

Fig. 2. Standard chemotaxis assay used for testing the worms’ chemotaxis to undiluted benzaldehyde.

Statistical Analysis
All chemotaxis indices were plotted as the mean ± standard error of the means, and were calculated from nine undiluted benzaldehyde conditioning assay test plates. Multiple group comparisons were performed with an ANOVA followed by a two-sample T test. Significance was set at $P < 0.05$ (two-tailed) (Sellings et al., 2013). After analysis, data was normal. The ANOVA test allowed us to examine the difference between multiple groups, but didn’t tell which group was different. Therefore, we used a 2 sample t-test to examine the difference between two groups to determine which groups showed significant difference from one another

RESULTS
In the experiment, we quantified the behavior of adult *C. elegans* under the influence of nicotine. After conditioning for 0-60 minutes in the presence of 24 µL undiluted benzaldehyde and 10 µL 50 mM nicotine, worms were placed on chemotaxis agar in the presence of 1 µL undiluted benzaldehyde. Each conditioning time was run in n=9 for a total of n=36. Under these conditions; one-way ANOVA $F = 19.43 \ P = 0.000; \ n = 36$; Fig. 3). One-way ANOVA showed a significant difference between conditioning and non-conditioning groups. In other words, worms that were conditioned between 15-60 minutes in the presence of both undiluted
benzaldehyde and nicotine showed a significant difference in CI value than worms that were not conditioned before the chemotaxis assay.

FIG. 3. Effects of Nicotine on CI when Paired with a Repellent. Chemotaxis assays were run 9 times for each of the 0-60 minute time intervals. One-Way ANOVA showed significant difference (p=0.00) between conditioning and non-conditioning groups.

To examine whether there were significant differences between two groups, we ran a 2-sample t-test. The test was run using data on number of worms at the test spot only at every 15-minute increment of the chemotaxis assay for each conditioning group. The numbers represent the amount of time in minutes of conditioning. (Two-Sample T-Test 0 vs. 15; P = 0.001; n=8; 0 vs. 30; P = 0.000; n=8; 15 vs. 30; P = 0.000; n=8, Fig. 4.)
FIG. 4. Effects of Nicotine on Worm Kinetics towards Repellent after Paired Conditioning. Two-Sample T-Test showed significant difference between non-conditioned worms and 15 minute and 30 minute conditioned worms. There was also significant difference between worms conditioned for 15 minutes and 30 minutes.

After group pairing assessment, the 2-Sample T-Test showed a significant difference between three groups. There was a significant difference between non-conditioned worms and worms conditioned for 15 minutes and worms conditioned for 30 minutes. In addition, there was a significant difference between worms conditioned for 15 minutes and 30 minutes.

DISCUSSION

Our study examined the influence of nicotine on *C. elegans* chemotactic behavior toward a known repellent: undiluted benzaldehyde. Worms were conditioned in the presence of nicotine and the repellent for times between 15 and 60 minutes or not conditioned at all. Statistical analysis showed there was a significant difference between nicotine-conditioned and non-conditioned groups in approach to undiluted benzaldehyde. Further statistical analysis examined the difference between groups conditioned for different lengths of time. There was statistically significant difference between worms that were not conditioned and worms that were conditioned for 15 or 30 minutes, as well as a difference between worms conditioned for 15 and 30 minutes.

After completing statistical analyses on the data and finding several cases of statistical significance, we cannot conclude that worms were attracted to undiluted benzaldehyde, as
defined by a positive CI value. However, the results show the worms became less repulsed to the repellent and appear to be developing a tolerance for it based on the differences in CI value and number of worms at the test spot during the assay for each of the conditioned groups. These results follow the trends from previous research indicating nicotine acts as a reward system.

There are several directions this research could go in the future. To begin, one could examine the effects of nicotine on chemotaxis under a few different conditions: paired in higher concentrations of nicotine, exposure to nicotine in larger quantities, or using a larger circle around the test and control spots to get a better idea of where the worms are on the plate as time passes on the chemotaxis assay.

Looking specifically at the acetylcholine receptors, nicotinic receptors are ligand-gated ion channels (McDowall, 2005). They are localized at neuromuscular junctions and in the central nervous system. When acetylcholine binds to the nicotinic receptors, they become activated, which brings the changes in arousal, reward, and motor control associated with nicotine use. Based on Sellings’ result demonstrating reduced approach to nicotine in acr mutants, we can assume the function of these receptors is inhibited in the mutant strains. Therefore, one could continue doing these experiments with the addition of mutants, specifically the acetylcholine mutants discussed previously and in Sellings et al. research.

LITERATURE CITED


