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Behavioral And Neurophysiological Examination Of The *C. elegans* Neuromuscular Junction

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Abstract

Caenorhabditis elegans is a model organism with well-defined neurophysiological characteristics. Movement in *C. elegans* is mediated through concurrent contraction on one side of the body and relaxation on the other at the neuromuscular junction (NMJ). Contraction and relaxation are mediated through cholinergic and GABAergic signaling respectively. The goal of the current study was to further elucidate these mechanisms of mobility via exposure to toluene, which produces mobility impairment in mice, humans, and *C. elegans*. More specifically, we were able to find a working concentration of toluene that produced behavioral and neurophysiological changes in *C. elegans.* Acute 10-minute toluene exposure to our working concentration decreases overall motor responsiveness measured one hour later ($p<0.01$). As well, this concentration resulted in behavioral responsiveness in the majority of animals tested. Finally, exposure to our working concentration of toluene resulted in an increase in puncta in the dorsal nerve cord in $_{p}$*unc-47*::*GFP* transgenic worms ($p<0.05$). These results suggest that toluene exposure affects behavior and neurophysiology in *C. elegans.* Our neurophysiological data suggests that toluene may be acting pre-synaptically. Currently we are using the concentration found in this study to further elucidate the action of toluene in *C. elegans.* We are also examining the affects of nicotine, which acts at cholinergic receptors at the NMJ, both independently and co-applied with toluene to better understand the balance between cholinergic and GABAergic signaling.

Keywords: Toluene, *C. elegans,* Nicotine

1. Introduction

Toluene is an industrial solvent which is commonly abused as a recreational drug.[1] Behaviorally, toluene can cause euphoria, dizziness and slurred speech.[2] It has been reported, that toluene acts on the γ- aminobutyric acid (GABA) neurotransmitter system in mice.[3] Furthermore, toluene has been shown to act on the GABAergic system in the nematode *C. elegans*.[4] When exposed to toluene *C. elegans* also show limited mobility.[5] In the current study, we worked to further elucidate the behavioral and neurophysiological effects of toluene in *C. elegans*.

C. elegans is a microscopic nematode with a well-defined nervous system. There are 302 neurons that make ~9000 connections.[6] Movement in *C. elegans* is mediated through concurrent contraction on one side of the body and relaxation on the other. This contraction and relaxation is mediated through cholinergic and GABAergic signaling respectively.[7] We took advantage of this preexisting motor system to look at the effects of toluene at the neuromuscular junction (NMJ) in *C. elegans*. Here, we present behavioral data that established and confirmed the necessary toluene exposure level sufficient to produce deficits in mobility seen with toluene in other model organisms.[3] Furthermore, morphological analyses of the *C. elegans* GABAergic motor neurons is also described allowing for more in-depth investigations of gene and protein expression in these neurons.

2. Experimental Procedures

2.1 Strains

Wild-type (N2) and EG1285 worms carrying transgene oxIs12 [$_{p}$*unc-47*::GFP + *lin-15*(+)] were originally acquired from the *Caenorhabditis* Genetics Center (University of Minnesota) and employed in these studies.

2.2 Drug Exposure and Behavior

Wild-type (N2) or transgenic *C. elegans* were cultivated on nematode growth medium (NGM) plates at 20 °C and age-synchronized by bleaching.[8] Behavioral testing occurred 72 hours after egg-laying. For toluene exposure, 20-30 worms were transferred to blank agar-filled petri plates and toluene (>99% grade, Sigma) was applied to the lid; the plate and lid were then sealed with double-wrapped Parafilm. Initial airborne concentrations were estimated using the Nelson Equation: 1 µl (13,000 ppm), 3 µl (38,000 ppm), 5 µl (64,000 ppm), 10 µl (127,000 ppm) and 15 µl (191,000 ppm) (Table 1; Equation 1).[9] These concentrations were considered to be over-estimations as toluene likely evaporated out of the plate both prior to sealing the plates and during the 10-minute exposure period. After toluene exposure, lids were removed and plates were left uncovered for either a 15-minute (short-term) or one-hour recovery period. Immediate effects of toluene were examined 15 minutes following exposure by responsiveness to head touch. To characterize the lasting effects of toluene, mobility was recorded after the one-hour recovery period using a camera (LifeCam, Microsoft) mounted to a dissecting microscope. Mobility was assessed in liquid by applying 5 µL of M9 buffer mixed with food coloring (for observer visualization) to an individual worm on an agar-filled petri plate; worm swimming behavior was recorded for 80 seconds. Swimming motions were analyzed by counting the total number of whole-body movement from one side to the other (body-bend, see below) for the 80 seconds of each test trial and averaged for each toluene exposure condition. For behavior analysis, coded video files were imported into Windows Movie Maker Data (Microsoft). Body-bends were defined as a single instance of a worm curving from one side to the opposite side and back again.

Table 1. Toluene dose in microliters and corresponding approximate PPM.

Toluene Dose in Microliters (µl)	Approximate Toluene Dose in PPM
1	13,000
3	38,000
5	64,000
10	127,000
15	191,000

A.
$$PPM = \frac{10^6 \times vL \times \rho L \times R \times T}{vD \times M \times P}$$

B.
$$64{,}000 = \frac{10^6 \times .005 \times .865 \times .082 \times 297.15}{15 \times 92.14 \times 1.15}$$

Equation 1. Sample Nelson equation.

A) Nelson Equation used to find airborne toluene concentration in PPM. Where vL is the volume of toluene placed on the lid of the plate, ρL is the density of toluene in g/ml, R is the gas constant, T is the temperature in K, vD is the

volume of the chamber in which the toluene is injected, M is the molecular weight of toluene, and P is pressure in atm.[9] B) An example of how the equation was used to estimate the PPM concentration of toluene when 5µl was injected onto the lid of the plate.

2.3 Microscopy

Confocal imaging of transgenic worms ($_{p}$*unc-47*::GFP) began at one hour after toluene exposure and continued for 3-4 hours. To prepare for imaging, worms were placed in 2,3-butanedione monoxime for paralysis (Sigma, CA) on either agar-filled welled microscope slides or glass-bottomed petri dishes. Images were captured on an inverted Olympus F1000 laser scanning confocal microscope using Fluoview software. Fifty to eighty micron sections were captured of the dorsal nerve cord opposite the DD2, VD3 and VD4 GABAergic motor neuron cell bodies.[6,10] Image capture settings were determined using the SETCOL look-up table to prevent pixel saturation and to ensure full representation of all gray levels. Images were quantified for size and area of expression with Image J (NIH); selection of pixels was determined by excluding all pixels in the bottom 25% of fluorescence intensity so as to operationalize the selection process and ensure background and auto-fluorescence were excluded. Measures were exported to Excel (Microsoft) and data were analyzed using SPSS.

2.4 Data Analysis

Resultant data was analyzed using *t*-tests for comparisons between two groups, univariate ANOVA for analyses with more than two groups. Tests of Least Significant Difference (LSD) were performed for post-hoc analyses. All data was analyzed using either SPSS (IBM) or GraphPad statistical software.

3. Results

3.1 Acute Exposure To High Concentrations Of Toluene Impairs *C. Elegans* Mobility

Brief exposure to toluene produced more unresponsive worms at higher toluene concentrations confirming results from previous studies.[11] Maximum immobility occurred with 15 µl toluene application (~191,000 ppm; Figure 1A). Both 5µl and 10µl toluene application also caused non-responsiveness at 15 minutes (Figure 1A). Movement generated on a solid substrate ("crawling") compared to movement generated in liquid media ("swimming") can be genetically differentiated.[12] Thus, to ensure paucity of behavior was due to immobility and not expiration, swimming behavior was also observed as placement in liquid media elicited motor responses from seemingly immobilized toluene-exposed worms. The number of "body-bends" (single swim motions) was significantly different between groups ($F(4, 36) = 6.806$, $p<0.001$). Worms exposed to higher toluene concentrations of ~64,000 ppm and ~127,000 ppm (5 µl and 10 µl toluene application, respectively) showed significantly lower rates of swim motion compared to non-exposed controls ($p<0.01$, see Figure 1B).

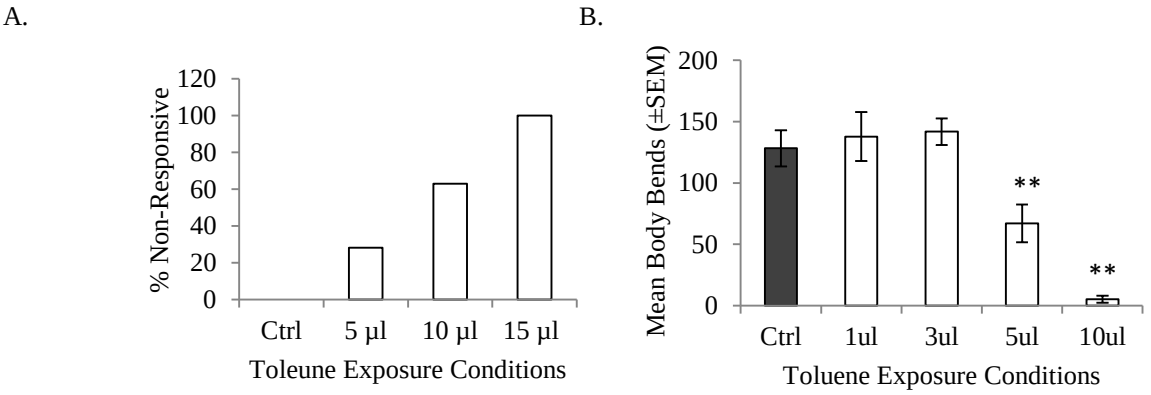

Figure 1. Increased toluene exposure results in progressive mobility impairment.

A) Percentage of worms non-responsive to head touch as a function of the total number of worms tested 15 minutes following exposure. B) Mean number of body-bends performed in an 80-second swimming test trial (±SEM) with 5 µl (~64,000 ppm) and 10 µl (~127,000 ppm) toluene showed significantly fewer body-bends compared to control and lower levels of toluene exposure. **p<0.01.

3.2 An Increased Number Of Presynaptic Contacts Results From Acute Toluene exposure

It has been shown previously that a $_{p}$*unc-47*::*GFP* transgenic strain can be employed to visualize the morphology of GABA neurons in *C. elegans.*[13] Confocal imaging of $_{p}$*unc-47*::*GFP* worms that express GFP in the *unc-47*, expressing GABAergic neurons was performed following toluene exposure to determine if an anatomical change in these neurons would result. The majority of GABAergic neurons are motor neurons with cell bodies that align along the ventral side of the worm in the ventral cord with processes that innervate muscle on both the ventral and dorsal sides.[14] A uniform region was observed on the dorsal side, directly opposite to the cell bodies of the DD2, VD3, and VD4 GABAergic motor neurons identified by their position from the nose of the worm.[15] GABAergic motor neurons synapse onto muscle in a fashion that is *en passant;* as such, GFP-expressing puncta along the dorsal and ventral nerve cords of $_{p}$*unc-47*::*GFP* transgenic worms are likely densities that represent *en passant* synapses (see Figure 2A arrowhead). Image analysis revealed a moderate increase in puncta number ($t(15) = 2.334$, $p<0.05$; see Figure 2B), but no difference in puncta area ($t(15) = .29$, $p>0.10$) following 5µl toluene exposure.

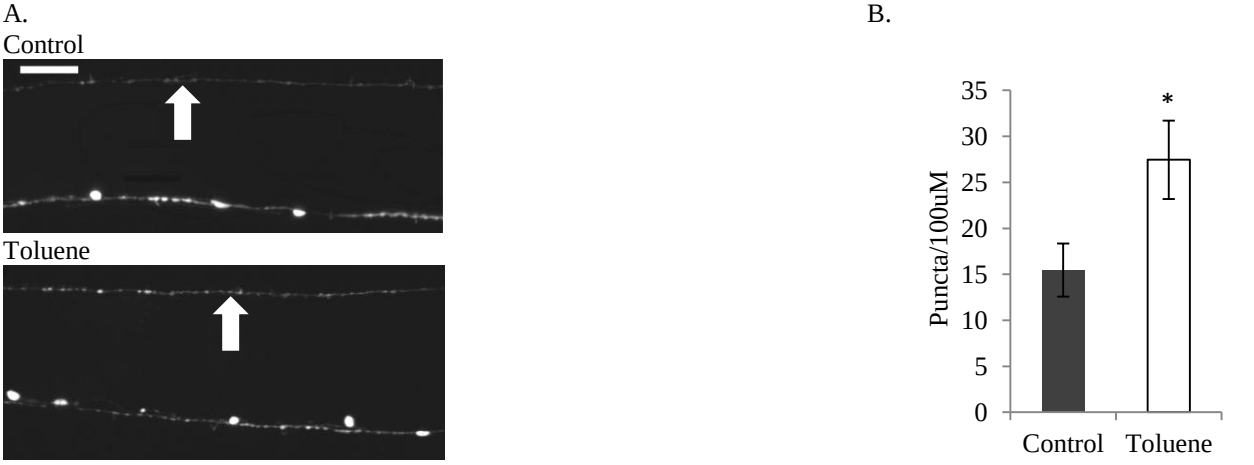

Figure 2. Alterations in GABA neuron morphology following toluene exposure.

A) Representative confocal images of GFP expressed in GABAergic motor neurons revealing puncta in the dorsal nerve cord (white arrowhead) at the terminal button directly opposite to cell bodies of the ventral nerve cord (above), B) 5µl toluene exposure resulted in an increase in puncta number with no change in puncta area, scale bar = 20 µM, *p<0.05.

4. Discussion

The results indicate a working concentration of 5µl (64,000 ppm) for toluene exposure that can be used to test both behavior and neuromorphological changes in *C. elegans*. At the 5µl (64,000ppm) concentration, worms are able to recover from toluene exposure although demonstrate reduced mobility as evidenced by the body bends data (Figure 1B); however, this concentration results in only minimal unresponsiveness 15 minutes after exposure (Figure 1A). This concentration is also appropriate as it resulted in measureable neuroanatomical changes in the GABAergic motor neurons affected by toluene exposure.

Confocal imaging revealed 5µl (64,000 ppm) toluene exposure results in more putative presynaptic GABA terminals in the GABAergic motor neurons (Figure 2B). An increase in presynaptic GABA terminals likely results in more GABAergic innervation of the muscle. This could lead to over-relaxation without the compensatory cholinergic contraction resulting in limited mobility. Additional studies are investigating the extent to which this neuroanatomical change reflects a neurophysiological change by examining expression of proteins involved in GABA signaling at the NMJ.

The muscle arms that receive motor neuron signals at the NMJ express both GABA and acetylcholine receptors.[7] If the relative immobility seen following toluene exposure is in fact due to increased GABA release, it is possible that altering activation of the acetylcholine receptors may modulate the behavioral effects of toluene. Ongoing studies are examining the cross effects of co-application of nicotine (agonist for the nicotinic acetylcholine receptors found at the *C. elegans* NMJ[16]) and toluene on mobility to determine if these drugs act in an opposing or synergistic fashion. Future studies will investigate if pathways involved in modulating surface nicotinic receptor density[17] similarly acts on neighboring GABA receptors at the NMJ. Understanding the effects of substances that act on receptors at the same synapses, as is found at the *C. elegans* NMJ, provides a unique opportunity to determine if and potentially by what mechanism(s), these signaling pathways cross-modulate one another.

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