Nicotine-Mediated Decreases in *Caenorhabditis elegans* Synaptic Puncta Area, Quantity, & Size

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Abstract

Chronic exposure to nicotine has long been associated with severe health risks, impacting not only smokers but also those exposed to secondhand smoke. This study explores the effects of chronic nicotine exposure during development on the behavior and neural development of *Caenorhabditis elegans* (*C. elegans*). By mimicking chronic exposure akin to long-term smoking conditions, a 200 μM nicotine concentration was administered to developing *C. elegans*. Our study assessed locomotion through head touch and thrashing assays, and used compound fluorescent microscope imaging techniques to examine synaptic puncta density and intensity in worms' synapses to quantify neurotransmission. Results revealed that locomotive responses did not differ between the groups, but the quantity of synaptic puncta decreased with application of 200 μM nicotine. We found mixed results regarding synaptic puncta intensity and area, with some significant results supporting our hypothesis and others refuting it. While behavioral responses did not align with predictions, the observed changes in synaptic puncta density suggested alterations in neural development attributable to nicotine exposure. Our study's limitations, such as using a single nicotine concentration and specific protein labeling, along with our mixed results, suggest the need for broader concentration studies and alternative protein markers.

Introduction

In 2021, the CDC estimated that nearly 12% of American adults smoked nicotine-containing cigarettes on a regular basis. Long-term smoking of tobacco products has been linked with several types of cancer and cardiovascular disease, with over 480,000 Americans dying from cigarette smoking-associated illnesses annually (CDC, 2023). Smoking not only

jeopardizes smokers' health, but also spouses and children of smokers via secondhand smoke exposure, as well as the next generation through a positive relationship between prenatal nicotine exposure and mortality rate (Smith *et al.,* 2013).

In addition to its implications in disease development, past literature shows a dose dependent relationship between nicotine exposure and *Caenorhabditis elegans* (*C. elegans*) locomotion (Sobkowiak *et al.,* 2011). *C. elegans,* the subjects of our experiment, are transparent nematodes and a model organism often used in the study of abused substances' mechanisms of action due to their completely mapped neural network, short life cycle, and hermaphrodite status (Smith *et al.,* 2013). In *C. elegans*, nicotine acts as an agonist to acetylcholine receptors (nAChRs), involved in the neuromuscular junction and the mesolimbic dopamine system, implicating nicotine in both locomotion – decreasing response to head touch and increasing assumed rigidity or a boomerang-shaped body line – and addiction/dependence (Smith *et al.,* 2013, Feng *et al.,* 2006).

Past literature has established that exposure to nicotine in *C. elegans* causes similar behavioral responses to exposure in mammals, including humans, rendering them a perfect organism to act as a proxy for human nicotine exposure (Sobkowiak *et al.,* 2011). Thus, our experiment sought to mimic chronic exposure conditions found in long-term smokers through examining the impacts of chronic exposure to nicotine in developing *C. elegans*. Nicotine exposure in humans occurs at low dosages for prolonged periods of time, with nicotine blood concentration ranging from 30 to 300 μM in habitual smokers (Smith *et al.,* 2013). Additionally, forced nicotine-mediated activation of nAChRs in mice (another mammal) changes long term potentiation (LTP) – a process thought to be involved in establishing neural circuits and learning – to short term potentiation (STP) (Placzek *et al.,* 2009). With these conditions in mind, by

exposing a time synchronized group of eggs to a medium containing 200 μM nicotine relative to a control group with no nicotine so these populations developed into adult worms whilst being exposed to their respective experimental conditions, we sought to find relationships between chronic nicotine exposure and behavioral responses and neurodevelopment. Specifically, we examined two behavioral assays: head touch assay and thrashing assay in M9 buffer, while seeking a synapse-level explanation for these behaviors by observing the density and intensity of synaptic puncta. We achieved this through labeling en passant synapses' (which innervate acetylcholinergic dorsal A-type (DA) motor neurons) synaptobrevin with green fluorescent protein (GFP), which can be observed under a fluorescent compound microscope. This synaptobrevin labeling would provide us with a better understanding of the cellular changes underlying observations in our behavioral assays. If nicotine is an agonist to nAChRs, jeopardizing neurodevelopment and decreasing locomotion, and our experimental group of *C. elegans* is exposed to 200μM of nicotine during development, then we predict decreased locomotive responses to head touch assay, fewer thrashes in our thrash assay, and decreased numbers and intensity of synaptic puncta in our experimental group relative to our control group in a fixed area.

Materials

In our experiment, we used a fluorescent compound microscope, microscope glass slides and coverslips, a *C. elegans* strain with GFP-tagged synaptobrevin on DA motor neuron synapses, glass pasteur pipettes with rubber bulbs, P20 and P1000 pipettes, a stereomicroscope, M9 buffer, bleach solution made from: [5mL bleach, 10mL 1M NaOH, and 15mL of water], 200 μM solution of nicotine in M9 buffer, a rotating platform, an AmScope microscope camera, a

fluorescent compound microscope, ImageJ software, and a mini centrifuge (Schuessler *et al*., 2023, 85-102).

Methods

Plate Setup and Bleaching

To expose our *C. elegans* to nicotine treatment, we created a total of four plates, with two control and two experimental as outlined in the lab manual (Schuessler *et al.,* 2023, 99-100). For our experimental plates, we pipetted 100µL of 200µM nicotine solution diluted in M9 buffer to the center of the plates' bacterial lawns. To all of these plates, we pipetted either 2 or 3µL of synchronized *C. elegans* eggs, bleached and prepared as described in the lab manual (Schuessler *et al.,* 2023, 100). In an attempt to acquire approximately 100 eggs per plate in our experiment, we added either 3µL or 2µL of eggs to these four plates. This adjustment in egg volumes added accounted for the differing densities of *C. elegans* found in the provided plates of eggs/worms, designated arbitrarily as plates A and B. Control and experimental plates from 3µL of population A eggs had 140 and 70 eggs, respectively, while the control and the experiment plates from 2µL of population B had 170 and 180 eggs, respectively.

Slide Setup, Image and Behavioral Analysis

We waited for our *C. elegans* to develop in either control or experimental conditions for one week. For our synaptic assays, we prepared glass slides with *C. elegans* from our control and experimental B groups, respectively, after paralyzing them with 5µL of 500mM sodium azide. We then imaged the DA motor neuron synapses of individual worms from each condition (or, in one case, multiple worms adjacent to one another in solution), labeled with GFP-tagged

synaptobrevin, using the compound fluorescent microscope. We analyzed these images using ImageJ software, examining the area and intensity of synaptic puncta in a 50μm region for both control and experimental groups as outlined in the manual, modules 4A and 4B (Schuessler *et al.,* 2023, 94-97 & 101-102).

For behavioral assays, we examined the movement response of ten *C. elegans* from control and experimental A groups in response to head touch from an artificial hair as described in lab manual module 4A. Thrashing assays were then performed on three worms from both the experimental and control groups as described in lab manual 4A (Schuessler *et al.*, 2023, 90-92).

Results

Head Touch Assay

The head touch assay was first conducted to obtain a general sense of the motor capabilities of the control population of *C. elegans* versus the 200 μM nicotine condition. All ten control group worms responded to the head touch by moving backwards, away from the hair on the pipette tip (Table 1). The experimental condition worms largely exhibited the same behavior, but two out of the ten worms first moved backwards, then reversed direction soon after and began moving forwards again (Table 1).

Thrashing Assay

A two sample Student's t-test shows that the sample means in thrashes/min between the control and the 200μM nicotine condition are not significantly different ($p = 0.37$). We also observed that worms in the experimental condition exhibited curling and stretching movements that were not observed in the control population. Worm thrashing behavior in M9 buffer was

recorded for one minute using the AmScope digital camera extension on the microscope. A thrash is counted when a worm swings its head and tail to the same side simultaneously. Watching the recording, we counted the number of thrashes for three worms in each condition. This was repeated twice to obtain an averaged number of thrashes for the three worms from each condition. The control condition *C. elegans* exhibited an average of 154.50 thrashes/min, and the 200 μM condition worms had an average of 156.83 thrashes/min (Figure 1).

Synaptic Puncta Quantification

The numbers of synaptic puncta along the chosen 50 μm stretches in the control condition were 8 for plate A and 9 for plate B (Figure 3). The 200 μM nicotine condition showed 4 puncta for plate A and 6 for plate B. No statistical tests were conducted as only one region per worm was analyzed. The compound fluorescent microscope was used to take images of individual *C. elegans* (Figure 2). ImageJ software was used to quantify the number, area, and intensity of synaptic puncta along a 50 μm length of the dorsal side of each individual *C. elegans*. We imaged experimental and control worms from plates A and B to account for any difference between the two plates' populations.

There was no significant difference between the puncta areas of the control and experimental conditions of plate A ($p = 0.16$). For plate B, the control condition had significantly higher puncta areas than the experimental condition ($p = 0.033$). For each identified puncta, the area and intensity were calculated using ImageJ by manually selecting around each puncta. The area is measured by the number of pixels within the selected area. The average areas for synaptic puncta in worms in the control condition were 13.50 and 25.22 for plates A and B, respectively (Figure 4). The average areas for the 200 μM nicotine condition were 16.25 and 14.33,

respectively. Two 2-sample Student's t-tests were conducted to compare the control and experimental condition of plate A and plate B separately.

The intensity of synaptic puncta in the control condition of plate A were significantly higher than in the experimental condition ($p = 0.0038$). For plate B, the intensity of synaptic puncta in the control condition were significantly lower than in the experimental condition ($p = 0.0018$). Intensity of the synaptic puncta was measured in arbitrary units by taking the brightness intensity of the synaptic puncta and subtracting the background intensity. The average intensities of synaptic puncta of worms in the control condition were 62.86 for plate A and 43.50 for plate B. For the 200 μM nicotine condition, the average intensities were 51.78 and 62.98 for plates A and B respectively (Figure 5). 2-sample Student's t-tests were conducted in the same manner as in the synaptic puncta area analysis.

Discussion

We initially hypothesized that chronic exposure to 200 μM of nicotine in developing *C. elegans* would lead to decreased numbers and intensity of synaptic puncta, decreased locomotive response to head touch assay, and fewer thrashes in thrash assay. While we observed a decreased number of synaptic puncta relative to control, the other three parts of this four-pronged hypothesis were not supported by the results. In this section, we will address these apparent inconsistencies in terms of past literature.

Regarding decreased numbers of synaptic puncta, our results were consistent with our prediction, based on existing literature which investigated the effects of nicotine exposure on gene expression in *C. elegans*. Fewer synaptic puncta in a fixed area under a compound fluorescent microscope implies a decrease in the amount of synapses innervating the body wall

muscle. The mechanism by which this decrease occurred may be related to low-to-moderate exposure to nicotine playing a regulating role in changing gene expression of genes implicated in cholinergic signaling (Smith *et. al*, 2013). Placzek *et al*. (2009) found that the timing of acetylcholine binding to nAChRs and electrical stimulation significantly altered synaptic potentiation in the hippocampus; specifically, long term depression instead of long term potentiation occurred when ACh was applied less than one second prior to stimulation. Due to the constant presence of nicotine in the development of the experimental *C. elegans* group, nAChRs were constantly being stimulated, so it can be assumed that there would be less than one second between nAChR binding and ongoing neural activity. Thus, the decreased number of synaptic puncta observed can be attributed to long term depression. This decrease in numbers of synaptic puncta in our experiment has implications for the development of human neural circuitry, as our experiment sought to mimic nicotinic conditions in human smokers, and *C. elegans*' response to nicotine has been shown to be analogous to humans' responses (Smith *et. al*, 2013, Sobkowiak *et. al*, 2011). This is pertinent especially for adolescents, with more than 6.6% of middle schoolers and 12.6% of high schoolers reporting current use of a tobacco product, as they are still undergoing building of neural circuitry, which could be adversely impacted by chronic nicotine exposure (CDC, 2023).

While we broadly predicted a decreased response to head touch assay, based on Smith *et. al,* 2013, we were somewhat surprised to see all experimental worms exhibiting responses similar to control worms, with one fifth of experimental worms exhibiting hyper-locomotive responses (i.e., moving backwards then forwards). While past literature has established a dose-dependent relationship between increased rates of no response to head touch, their experiments are not exactly analogous to ours in terms of method of exposure or testing. Past studies

performed head touch assays on over 600 worms exposed to nicotine concentrations for 24 hours as fully-developed worms (Smith *et. al,* 2013). Our experiment, in contrast to this acute exposure to developed worms, utilized a chronic exposure to developing worms. The apparent behavioral discrepancy can be explained in multiple ways. Firstly, if nicotine changed the gene expression for genes implicated in cholinergic signaling of our developing *C. elegans*, as shown in Smith *et. al,* 2013, their responses would likely differ from developed *C. elegans* acutely exposed to nicotine, as those worms' neural circuitry would likely be fully-formed. In the case of the developed *C. elegans* exposed to nicotine, the compound could simply act as an agonist to nAChRs, decreasing locomotion, while worms exposed to nicotine in development could have fundamentally different circuitry. Additionally, long-term exposure to nicotine in *C. elegans* has been shown to elicit a tolerance effect (Feng *et. al,* 2006). This tolerance impact could be observed in our chronically exposed worms through their locomotive responses to head touch while absent in the acutely exposed worms of past studies.

While we predicted a decrease in the number of thrashes during our one-minute thrashing assay, we observed a non-statistically significant increase in the number of thrashes in our experimental relative to our control group. Our prediction was primarily based on findings that response to head touch assay decreased in a dose-dependent manner upon exposure to nicotine (Smith *et. al,* 2013). We assumed that the thrashing behavioral assay would follow similar patterns. However, our findings corroborate literature showing that moderate concentrations of nicotine (100μM-200μM) actually increased the mean speed of locomotion in *C. elegans*, accomplished via thrashing (Sobkowiak *et. al,* 2011). Additionally, we observed our worms displaying a rigid or boomerang-shaped body line during thrashes, consistent with findings from Smith *et. al,* 2013.

Finally, our prediction that exposure to nicotine would decrease puncta brightness intensity was observed in analysis of worms from plate A, but in plate B the experimental group had a greater mean intensity than the corresponding control group, with both of these findings being statistically significant. A possible explanation for this discrepancy is the difference in relative brightness of the backgrounds. The mean intensity of our puncta was calculated by recording the intensity of individual puncta, then subtracting a representative background intensity from all of them and averaging the result, as to account for inter-image exposure differences. Images of worms from plate A both had a similarly dark background, with intensities of 38.401 and 35.597. Images from plate B, however, had vastly different background intensities of 57.543 for the control and 38.962 for the experimental. This unusually high background intensity for the control B image is due to the presence of adjacent, fluorescent worms, which made it difficult to select a representative area of background similar to the other images (Figure 2b). A higher background intensity would lead to an artificially low mean intensity of our control, explaining this anomalous result.

Future studies could involve investigating the impacts of multiple concentrations of nicotine, much like Sobkowiak *et. al,* 2011, which examined concentrations and their behavioral effects ranging from 1μM to 30mM. However, their study examined acute exposure impacts, so a study that examined a range of concentrations as broad as theirs in a time course similar to ours would be informative in terms of a possible dose-dependent impact on development. This relationship could be established in an imaging manner similar to ours, with possible correlations between nicotine concentration exposure and synaptic puncta prevalence, density, and intensity being further elucidated by a gradient of images resulting from multiple concentrations (as opposed to a binary control versus experimental analysis). Another future study could examine

changes in gene expression outlined in Smith *et. al*, 2013 by GFP-labeling nAChRs as opposed to synaptobrevin generally. This experiment shifts from focusing on a general protein (synaptobrevin) to a specific receptor implicated in nicotine treatment (nAChRs). If performed with multiple concentrations of nicotine as outlined earlier in this paragraph, the experiment could provide a more in-depth view of changes in gene expression on a subcellular level.

In summary, we found data that both supported and failed to support our original prediction regarding nicotine's impacts on *C. elegans* synapses and behaviors. While a decreased number of synaptic puncta was observed, our behavioral assays and intensity analysis reveal a more complex interaction between nicotine and the nervous system than we initially anticipated. Our study's scope was limited by our decision to only examine one experimental concentration of nicotine, which also limits our analysis of this nuanced relationship between neural development and chronic exposure to nicotine. Further studies should be conducted with more concentrations of nicotine and/or with differently and more specifically labeled proteins to address the primary limitation of our study and further uncover this relationship. The continued study of nicotine's impacts on *C. elegans* nervous system remains relevant and scientifically significant due to it being an easily accessible model organism whose interactions with nicotine can serve as a proxy for humans' interactions, and thus provide a pathway to improving understanding of molecular and cellular mechanisms of nicotine's impacts on the human body and society.

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Figures and Tables

Table 1. Observed movement in response to head touch for *C. elegans* exposed to control versus 200 μM nicotine

C. elegans number	Control A	200 μM Nicotine
1	Moved backwards	Moved backwards
2	Moved backwards	Moved backwards
3	Moved backwards	Moved backwards
4	Moved backwards	Moved backwards then reversed direction
5	Moved backwards	Moved backwards
6	Moved backwards	Moved backwards
7	Moved backwards	Moved backwards
8	Moved backwards	Moved backwards then reversed direction
9	Moved backwards	Moved backwards
10	Moved backwards	Moved backwards

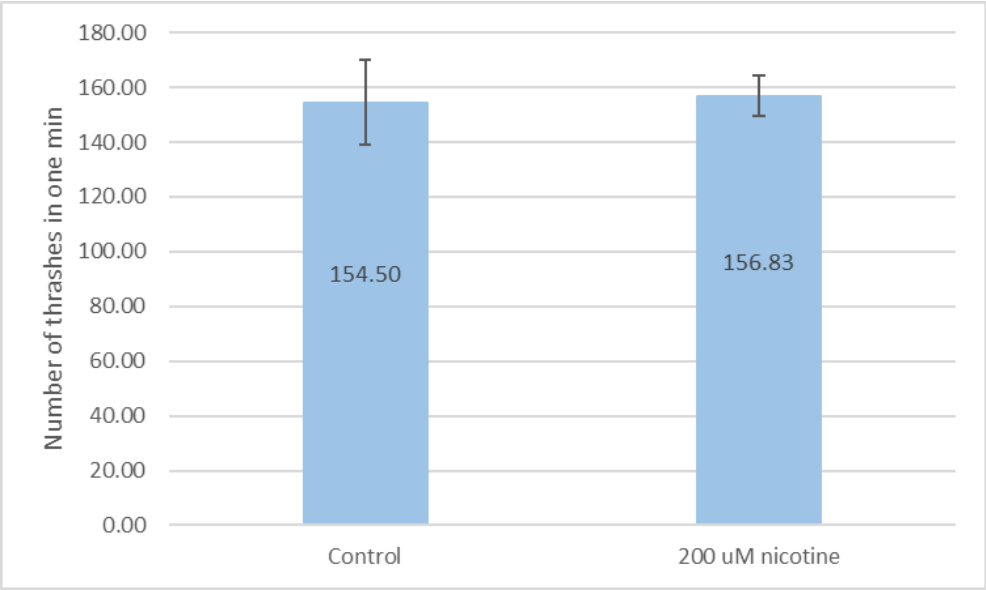

Figure 1. Graph of the average number of thrashes per minute displayed by 3 representative *C. elegans* from the 200 µM nicotine condition and control condition. The left bar displays the average number of thrashes per minute of 3 worms that developed in the control growth plate. The right bar shows the average number of thrashes per minute of 3 worms that developed in the 200 µM nicotine growth plate. Error bars represent the standard deviation. A two sample 1-tailed homoscedastic Student's t-test shows that there is no significant difference between the control and experimental groups, $p = 0.37$.

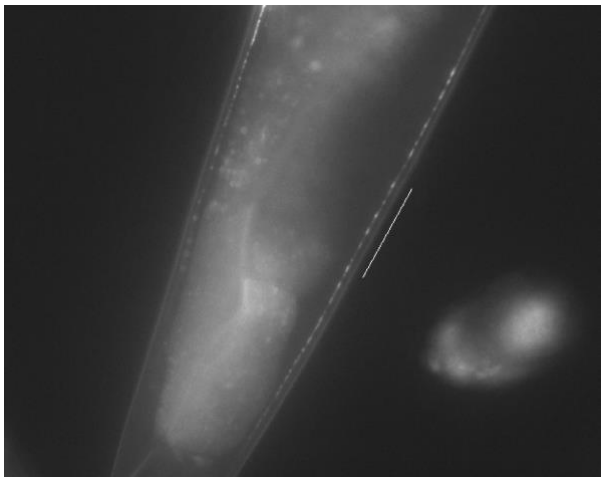

Figure 2a. Image of *C. elegans* (A) from the control condition.

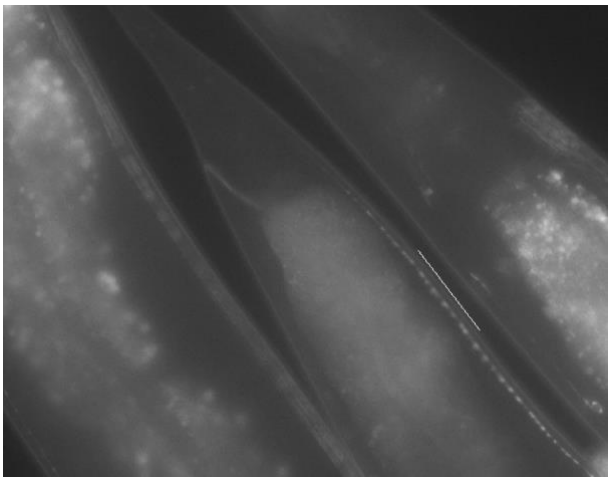

Figure 2b. Image of *C. elegans* (B) from the control condition.

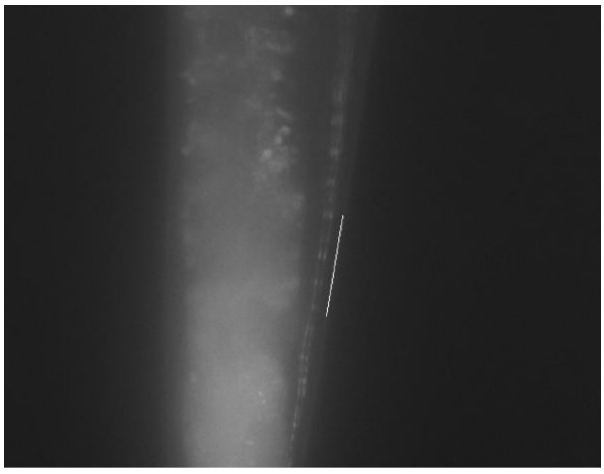

Figure 2c. Image of *C. elegans* (A) from the 200 μM nicotine condition.

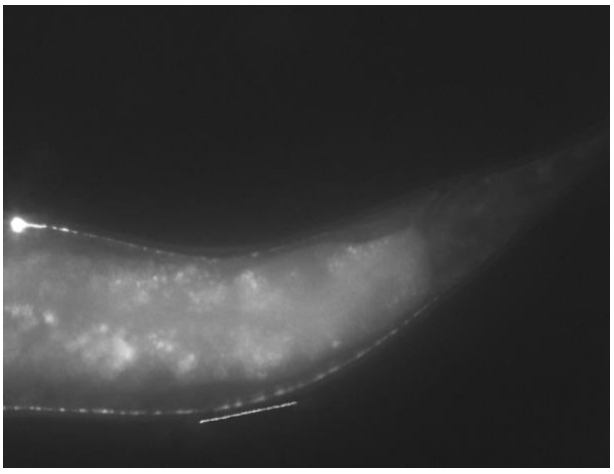

Figure 2d. Image of *C. elegans* (B) from the 200 μM nicotine condition.

Figure 2. Images of *C. elegans* taken with compound fluorescent microscope. The synaptic puncta appear as bright dots along the dorsal and ventral sides of the worm. The 50 μm (105 pixel) line drawn beside the dorsal side is the region containing the puncta that were analyzed.

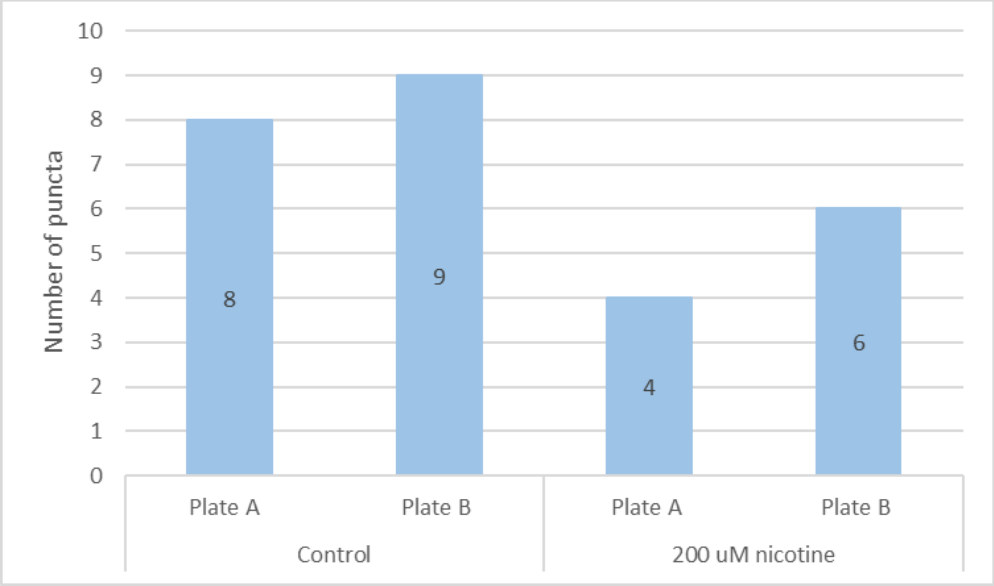

Figure 3. Number of synaptic puncta in one *C. elegans* from plates A and B in both the 200 µM nicotine condition and control condition. The number of synaptic puncta for all worms were measured within a representative 50 µm length along the dorsal side of *C. elegans*. The two bars on the left represent the number of synaptic puncta of one worm each in control plates A and B. The two bars on the right represent the number of synaptic puncta of one worm each in 200 µM nicotine plates A and B.

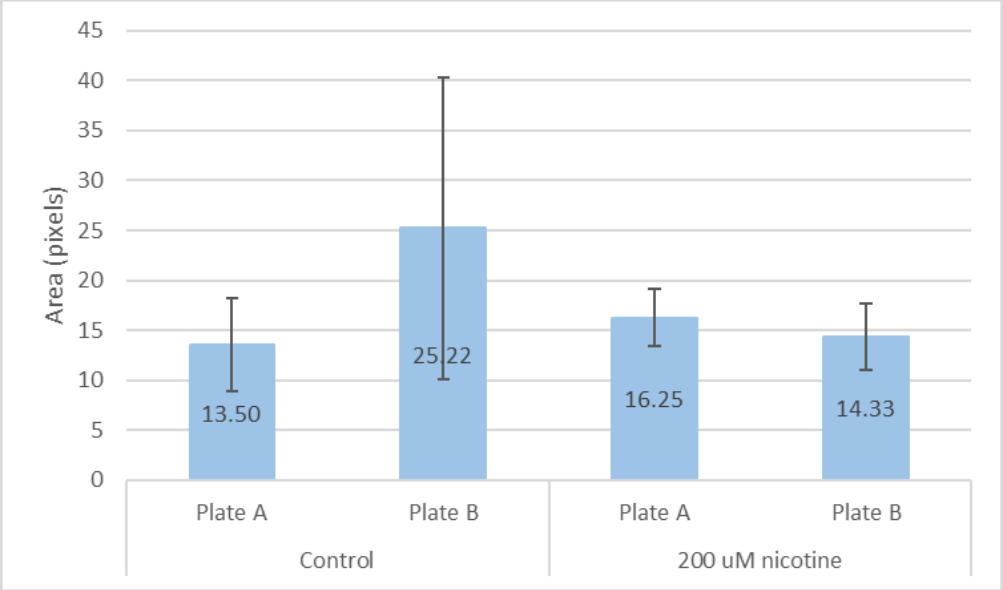

Figure 4. Average area of synaptic puncta measured in pixels in plates A and B of both the 200 µM nicotine condition and control condition. The synaptic puncta measured were within a representative 50 µm length along the dorsal side of *C. elegans*. The two bars on the left display the average areas of synaptic puncta from one worm each in control growth plates A and B. The two bars on the right show the average areas of synaptic puncta from one worm each in 200 µM nicotine growth plates A and B. Error bars represent the standard deviation. A two sample 1-tailed homoscedastic Student's t-test shows that there is no significant difference between the control and experimental group puncta areas for plate A, $p = 0.16$. A two sample 1-tailed heteroscedastic Student's t-test shows that the control condition displays significantly larger puncta areas than the experimental condition for plate B, $p = 0.033$.

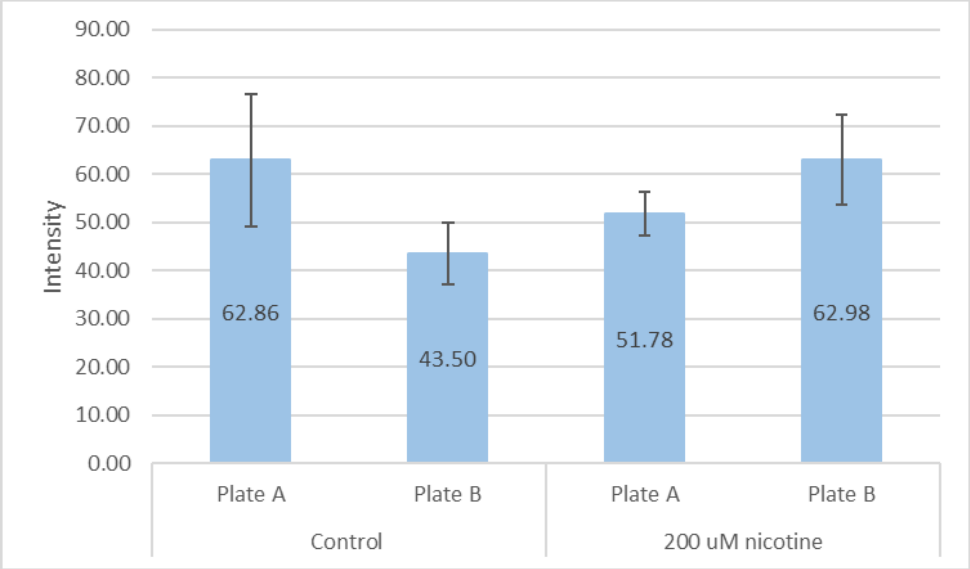

Figure 5. Average brightness intensity of synaptic puncta measured in arbitrary units of plates A and B in both the 200 μM nicotine condition and control condition. The synaptic puncta measured were within a representative 50 μm length along the dorsal side of *C. elegans*. The two bars on the left display the average intensity of synaptic puncta of one worm each from control growth plates A and B. The two bars on the right show the average intensity of synaptic puncta of one worm each in 200 μM nicotine growth plates A and B. Error bars represent the standard deviation. A two sample 1-tailed heteroscedastic Student's t-test shows that the control condition synaptic puncta were significantly greater in intensity than the experimental condition for plate A, $p = 0.0038$. A two sample 1-tailed homoscedastic Student's t-test shows that the control condition displays significantly smaller puncta areas than the experimental condition, $p = 0.0018$.