ARE THERE NICOTINIC ACETYLCHOLINE RECEPTORS ON PHRENIC MOTOR NEURONS?

By

KIMBERLY HENDERSON

A Thesis Submitted to The Honors College

In Partial Fulfillment of the Bachelors Degree
With Honors in

Neuroscience and Cognitive Science

THE UNIVERSITY OF ARIZONA

AUGUST 2019

Approved by:

Dr. Ralph F. Fregosi
Department of Physiology

Abstract

Nicotinic acetylcholine receptors (nAChRs) are expressed throughout the central nervous system, including on neuron populations that control breathing. The specific locations of nAChRs on respiratory related neurons are relatively unknown and their presence on phrenic motor neurons (PMNs) could indicate a point at which developmental nicotine exposure may impact breathing. We hypothesize that application of nicotine to the PMNs will elicit changes in amplitude and area of respiratory motor bursting recorded from cervical 3-5 ventral roots due to the presence of nAChRs on PMNs. A brainstem spinal cord split-bath preparation was used to separately perfuse brainstem and spinal cord chambers with artificial cerebrospinal fluid (aCSF), and nicotinic aCSF was added to the spinal cord chamber. Burst amplitude and area under the curve were measured at baseline and during application of three different nicotine concentrations (400nM, 4μM, 40μM). Our results show that while 400nM nicotinic aCSF did not significantly affect the amplitude or area of bursts, both 4μM and 40 μM nicotinic aCSF caused an initial increase in amplitude and area of the bursts, indicating nAChR activation, followed by a decrease in these parameters, indicating nAChR desensitization. These findings indicate the presence of nAChR on PMNs or neurons that synapse with PMNs.

Keywords: Nicotine, nAChR, artificial Cerebrospinal Fluid (aCSF), Phrenic Nerve, Phrenic Motor Neurons, Brainstem-Spinal Cord Preparation

Introduction:

The respiratory control system is complex automatic system in the body that is powered by the brainstem. There are several factors that can create problems in the respiratory control system in both adults and babies, one in particular is smoking. Smoking during pregnancy results in respiratory issues in young children, especially babies. About half a million newborns born to smoking mothers each year and those who were exposed to nicotine in utero typically suffer from cardiovascular and respiratory issues or die from Sudden Infant Death Syndrome (SIDS).

Sudden infant death syndrome is the unexplained death of a seemingly healthy baby (Mayo Clinic). It usually happens during sleep to babies that are less than a year old (Mayo Clinic). Approximately 2,500 babies die of SIDS each year in the U.S. and it is currently the third leading cause of infant mortality *(Illinois Department of Public Health)*. One possible explanation for SIDS could be defects in the portion of an infant's brainstem that controls breathing. One reason for these defects could be brought on by developmental nicotine exposure.

*Nicotine Acetylcholine Receptors (nAChRs***)** are present ubiquitously throughout the central nervous system including brainstem and spinal cord

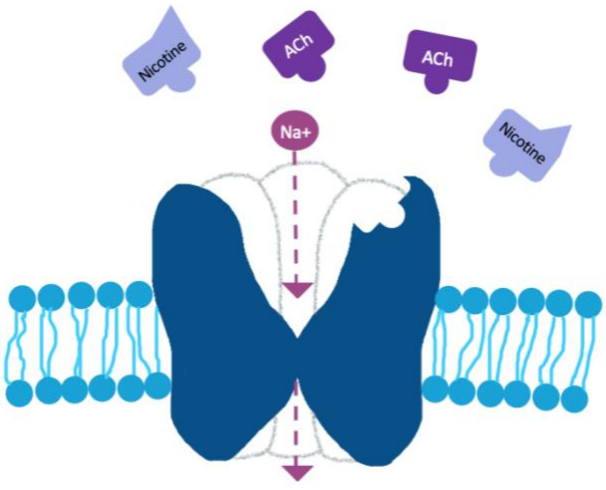

Figure 1*: Nicotine Acetylcholine Receptor*

regions involved in the control of breathing. (Shao and Feldman 2009) However, their specific locations in these regions are still relatively unknown. nAChRs are seven transmembrane domain protein receptors that bind either the neurotransmitter Acetylcholine or the addictive chemical nicotine. (Albuquerque 2009) Once acetylcholine or nicotine bind to the receptor a conformational change occurs allowing for an influx of ions, typically sodium. nAChRs activation can modulate respiratory pattern and these receptors mediate the effects of nicotine from tobacco smoke and e-cigarette usage. (Shao and Feldman 2009) An activation of nAChRs always causes depolarization, but their presence on both excitatory and inhibitory neurons can lead to an array of complex effects on physiology. (Albuquerque 2009)

The phrenic nerve innervates the diaphragm controlling its contraction and relaxation. The diaphragm is the muscle necessary for breathing as it is responsible for the movement of the thorax and lungs. The phrenic nerve is made up of the C3-C5 nerves, these nerves also go to other parts of the body such as the arm. Therefore, the activation of the phrenic motor neurons greatly effects respiratory activity, so if nAChR are present on phrenic motor neurons they can alter the activity when nicotine is introduced to the system. nAChRs are important for the modulation of fast-synaptic transmission, and if they are located on phrenic motors neurons (PMNs), or on other neurons that synapse with the PMNs, then their activation may impact neural output to the diaphragm. Thus, determining whether or not there are nAChR on phrenic motor neurons could indicate another way that nicotine can affect the respiratory system of an infant born to a smoking mother.

Materials and Methods:

Animals: Thirty neonatal rats (6.50-15g) of either sex were used, ranging from postnatal day 1-5 (P1-P5). Pups were derived from litters born to dams who drank saccharin water (1g saccharin/100mL tap water) throughout gestation and during the first week after spontaneous vaginal delivery. These dams were a part of a control group (saccharin water) for a larger experiment investigating the effects of developmental nicotine exposure, where nicotine was delivered to pregnant dams through drinking water using saccharin for palatability (nicotine + saccharin water). All data were obtained from experiments approved by the Institutional Animal Care and Use Committee at the University of Arizona.

Spinal Cord and Brainstem Extraction. P1-P5 neonatal rats were anesthetized with hypothermia. Once anesthetized, the brainstem and spinal cord were removed and placed in cold oxygenated (O_2, 95%; CO_2, 5%) artificial cerebrospinal fluid solution (aCSF) consisting of (in mM): 125 NaCl, 24 $NaHCO_3$, 1 $MgCl_2$, 2.5 KCl, 1 $CaCl_2$, and 10 D-glucose with a pH of 7.3-7.45 and osmolarity of 300-320 mOSM.

Electrophysiology. Once extracted, the brainstem-spinal cord preparation was placed in a split-bath recording chamber, which has a partition separating the brainstem and spinal cord portions of tissue so that the two chambers (rostral and caudal) can be perfused separately. Each chamber was filled with oxygenated aCSF with a constant drip flow. Glass micro pipettes

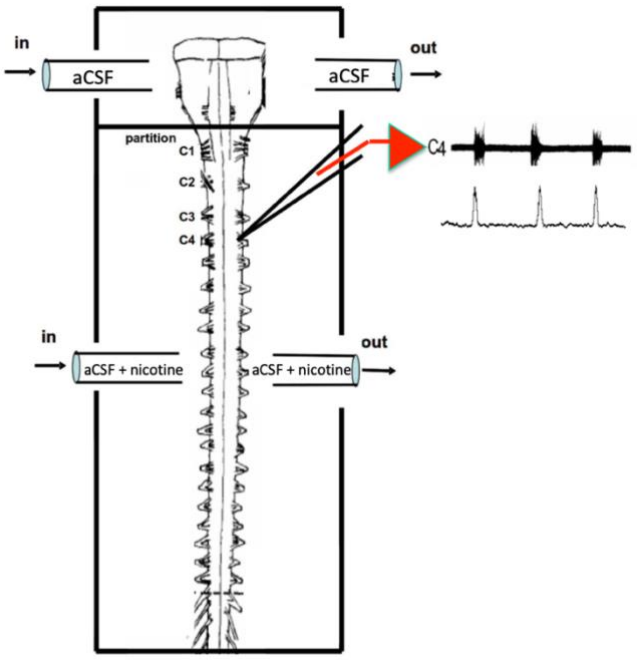

Figure 2: *Brainstem-spinal cord preparation in split-bath recording chamber with a partition separating the brainstem and spinal cord.*

containing a silver wire electrode were filled with aCSF and suction was used to record from cervical ventral roots 3-5 (C3-C5) which make up the phrenic nerve that innervates the diaphragm. From these ventral roots, spontaneous respiratory motor bursting can be recorded. Baseline amplitude of these respiratory bursts was recorded for 25 minutes with regular aCSF perfusing through both chambers. After the 25-minute baseline period, nicotinic aCSF was perfused into the caudal (spinal cord) chamber only. Spontaneous bursting was recorded throughout the twenty-minute exposure to nicotinic aCSF, and throughout a 25-minute period of "wash-out" with regular aCSF. Amplitude changes are closely monitored to determine the possible presence or absence of nAChRs. To record the bursting activity, a Grass Instrument Amplifier was used that filtered the signal with a bandpass ranging from 30-1000 Hz.

Nicotine Concentrations: Three sets of experiments were performed using three different concentrations of nicotine: 400nM, 4μM, and 40μM. Nicotinic aCSF was prepared daily by adding 50μL, 500μL or 5mL of stock nicotine solution (1mg/1mL Nicotine Bitartrate Dihydrate, M.W. 498.44/mol), respectively, to 250mL of oxygenated aCSF. To equilibrate, the nicotinic aCSF is oxygenated for about 10 minutes prior to being used for each experiment.

Data Analysis and Statistics: Three Spike2 Software (CED, Cambridge, UK) filter settings were used to filter bursting activity obtained from the Grass Instrument Amplifier*: DC remove*, *rectify*, and *smooth* with a 0.1sec time constant. The amplitude and area for each individual burst for both the 400nM and 4μM concentrations were calculated using a custom software program written with Spike2 Software. *Figure 3* shows how the data analysis was done using this software. For the 40μM data, a different analysis approach had to be used due to the amount of evoked tonic activity. The peak amplitude of tonic activity was compared to the baseline to determine the percentage change from baseline, shown in *Figure 4*.

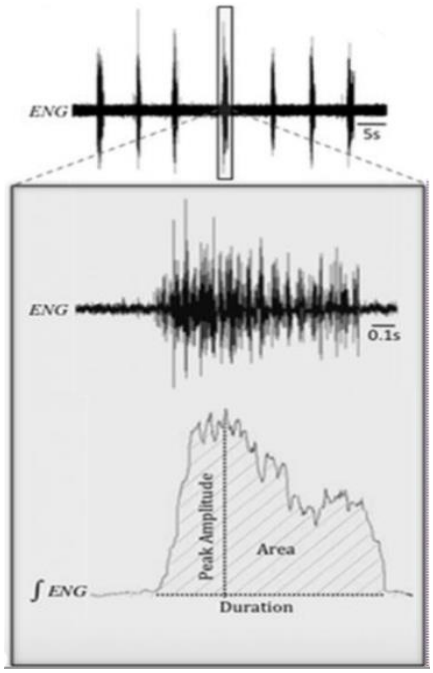

Figure 3*: Analysis method used to determine amplitude and area taken from each individual burst of the 400nM and 4*μM *doses. (Jaiswal, S. J., Pilarski, J.Q., Harrison, C.M., Fregosi, R.F.)*

Note that the baseline was considered 100%. One sample t-tests were used to determine if drug-induced changes were significant, using $P<0.05$ as the threshold for significance (GraphPad Prism 6 was used for all statistical analyses).

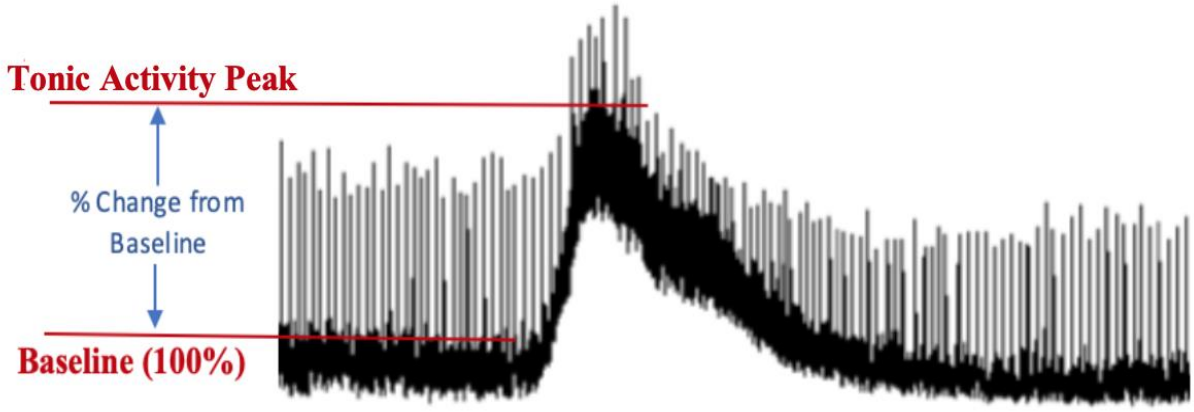

***Figure 4**: Data analysis method used for 40μM concentration raw data. Due to the large increases in tonic activity, the customized Spike2 program could not analyze the area under the curve nor the amplitude of each individual burst for this data set. The figure shows from which point baseline and the peak amplitude were measured from and what was considered to be the percentage change from baseline.*

Results:

400nM Nicotinic CSF (Low dose)

First, to test the effects of a low-dose of nicotine on respiratory burst amplitude, area and average amplitude, we perfused 400nM nicotinic aCSF into the spinal cord chamber of the split bath (caudal chamber). *Figure* 5 shows a representative recording from the low-dose nicotinic aCSF group, where the bottom trace is the raw record and top trace is the integrated and rectified record used for analysis. Baseline amplitude was recorded for 25 minutes, after which 400nM nicotinic aCSF was added to the spinal cord chamber of the split bath for 20 minutes. At this dose, the raw data showed a slight increase or no increase in amplitude and area during the nCSF phase. Upon washout, the amplitude and area decreased below baseline values or remained unchanged. Burst amplitude, area, and average amplitude were averaged over the last 20 minutes of the baseline period, and over the entirety of nicotine exposure and washout. Overall, the statistical analysis showed no significant change from baseline in either the nCSF phase or in the wash-out period. There was also no change in tonic activity throughout the 400nM dose experiments.

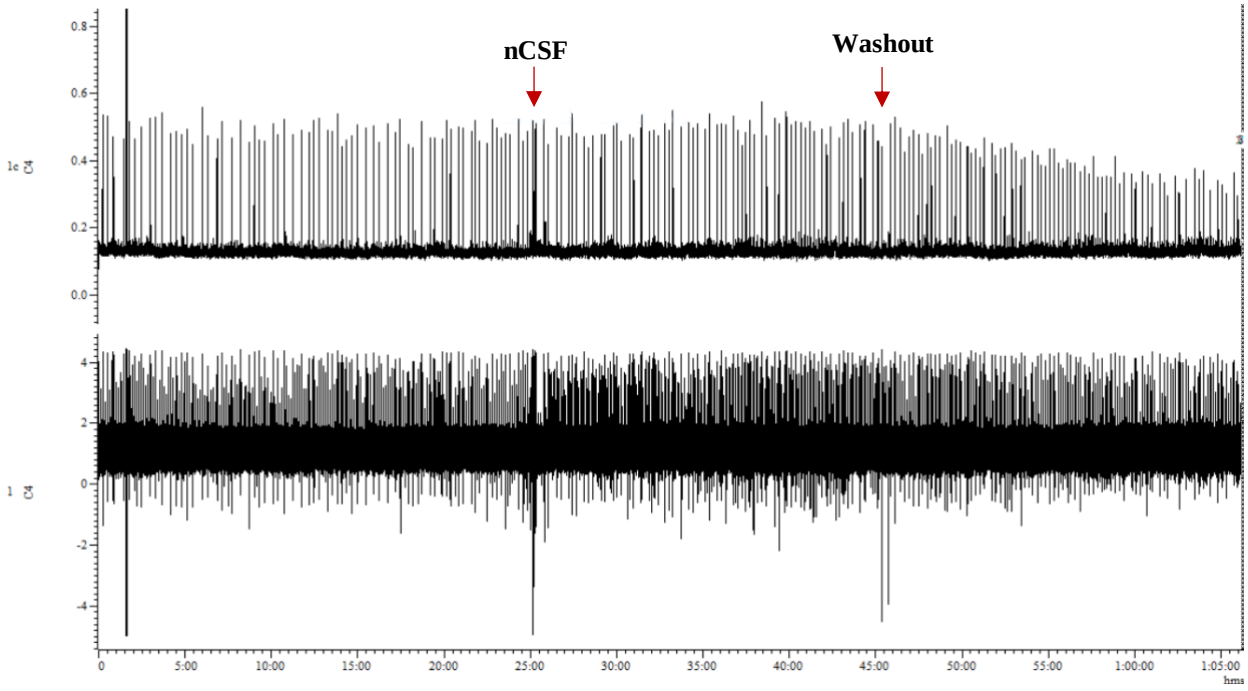

Figure 5*: Representative trace of raw record (bottom) and the rectified and smoothed record (Top) used for analysis. Arrows indicate the start and end of perfusion of low dose nicotinic aCSF (400nM) into the spinal cord chamber of the split bath.*

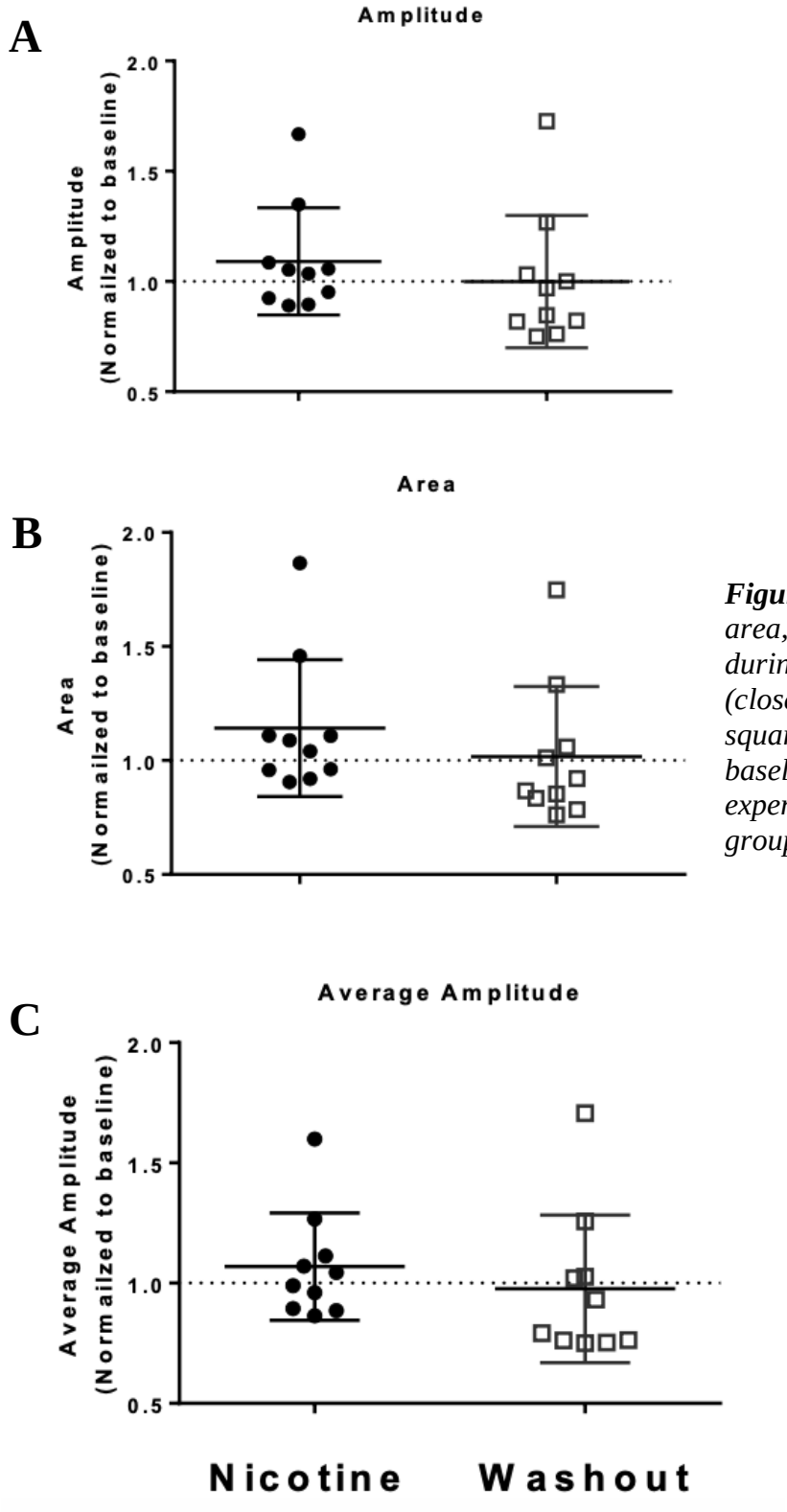

Figure 6*: Changes in burst amplitude, area, and average amplitude recorded during 400nM nicotinic aCSF application (closed circles) and after washout (open squares). Values are normalized to baseline. Each point represents a single experiment and horizontal lines indicate group mean ± SD.*

4 μM Nicotinic CSF (Middle dose)

Next, we tested a moderate dose of nicotine by perfusing the spinal cord chamber of the split bath with 4μM Nicotinic CSF. *Figure 6* shows a representative recording from the 4μM dose nicotinic aCSF group. With bath application of 4μM nicotinic CSF (nCSF) there were a statistically significant increases in amplitude, area, and average amplitude. As seen in Figure 7, the amplitude starts to increase at about 4-5 minutes after the introduction of the nicotinic CSF. One or two minutes after the initial increase, there is a notable decrease in burst amplitude after the initial peak, and amplitude returns to baseline by the end of the nCSF wash-in phase. We hypothesize that this decrease indicates desensitization of nAChRs. After 20 minutes in nCSF, washout began with regular aCSF began. During washout amplitude and area remained near baseline values. The statistical analysis showed a 20% increase from baseline amplitude with nCSF and a 4.9% increase compared to baseline during washout. The percentage increase of the washout meant that the amplitude dropped significantly after the nCSF phase but remained 4.9% above the baseline. A similar pattern was observed in both the area and average amplitude analysis. Area increased by 34% compared to baseline during nCSF phase and remained increased by 8% from baseline after washout (a 26% decrease from nCSF phase). The average amplitude increased by 17% from baseline during the nCSF phase and remained 4.7% increased from baseline during the washout (a 12.3% decrease from the nCSF).

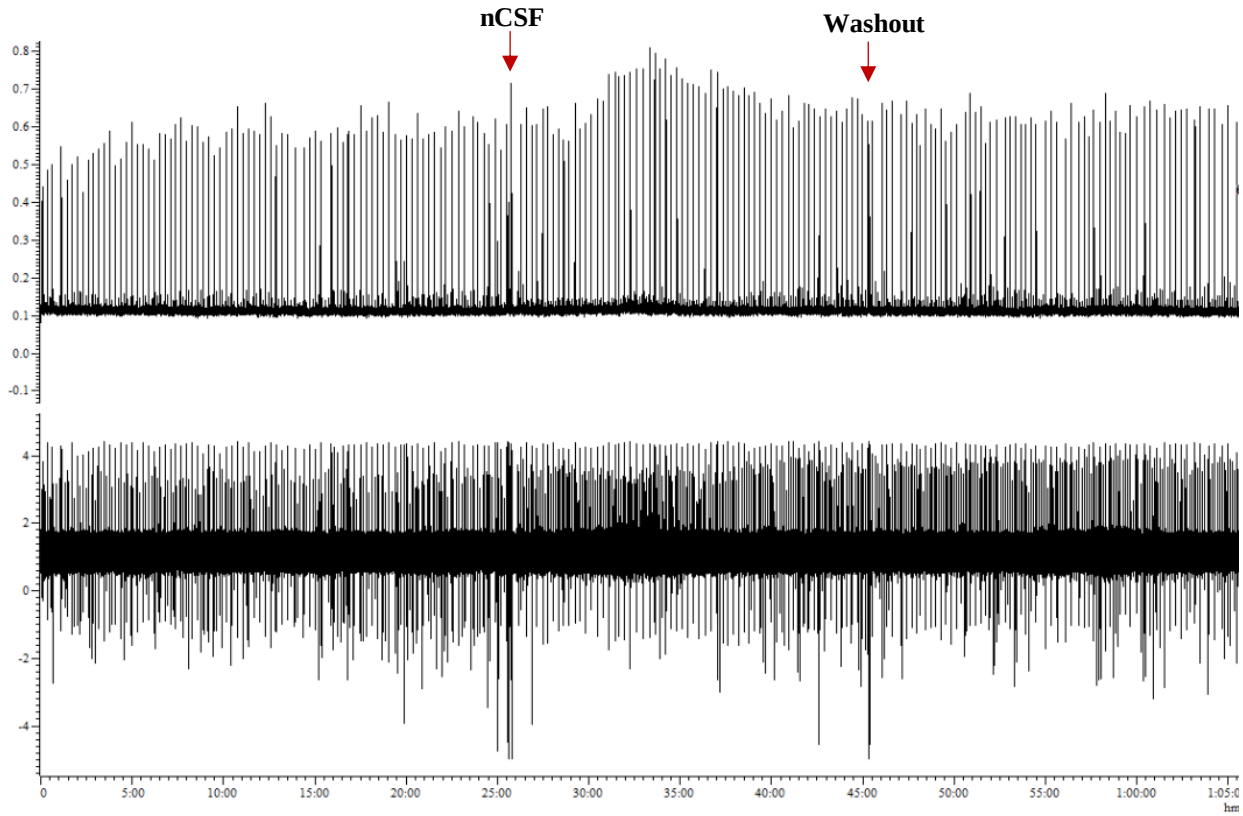

Figure 7*: *Figure 9*: *Representative trace of raw record (bottom) and the rectified and smoothed record (Top) used for analysis. Arrows indicate the start and end of perfusion of middle dose nicotinic aCSF (4μM) into the spinal cord chamber of the split bath.

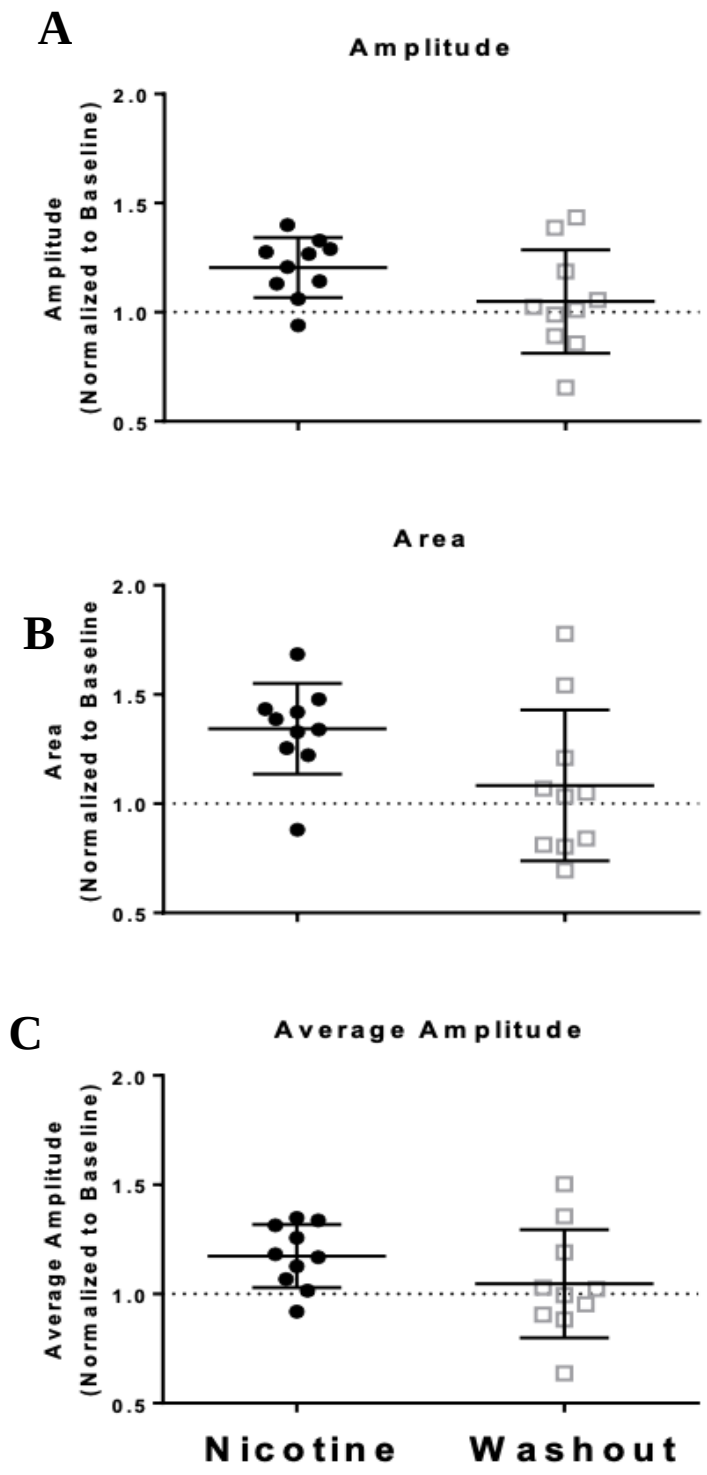

Figure 8: *Changes in burst amplitude, area, and average amplitude recorded during 4*μM *nicotinic aCSF application (closed circles) and after washout (open squares). Values are normalized to baseline. Each point represents a single experiment and horizontal lines indicate group mean ± SD.*

40μM Nicotinic CSF (High dose)

Lastly, we tested a high dose of nicotine by perfusing the spinal cord chamber of the split bath with 40 μM nicotinic CSF. A representative recording is shown in *Figure 9.* The application of 40μM nCSF caused a robust increase in amplitude, area and average amplitude. However, the exact percent increases of the area and amplitude were unable to be analyzed due to a concurrent increase in tonic activity. Instead, the percent increase from baseline to the peak of the tonic activity was measured. About three to four minutes after the nCSF is applied, there is a sharp increase in tonic activity. This is similar to the moderate dose of 4μM nCSF, however, the increase begins sooner with the higher dose. Figure 10A shows the average data for this group. Tonic activity increased 200%-800% from baseline. The total duration of the elevated tonic activity was 8 minutes on average, as shown in *Figure 10B*. Upon washout, the tonic activity decreased back to baseline about 5-6 minutes after the peak, once again indicating desensitization of the receptors.

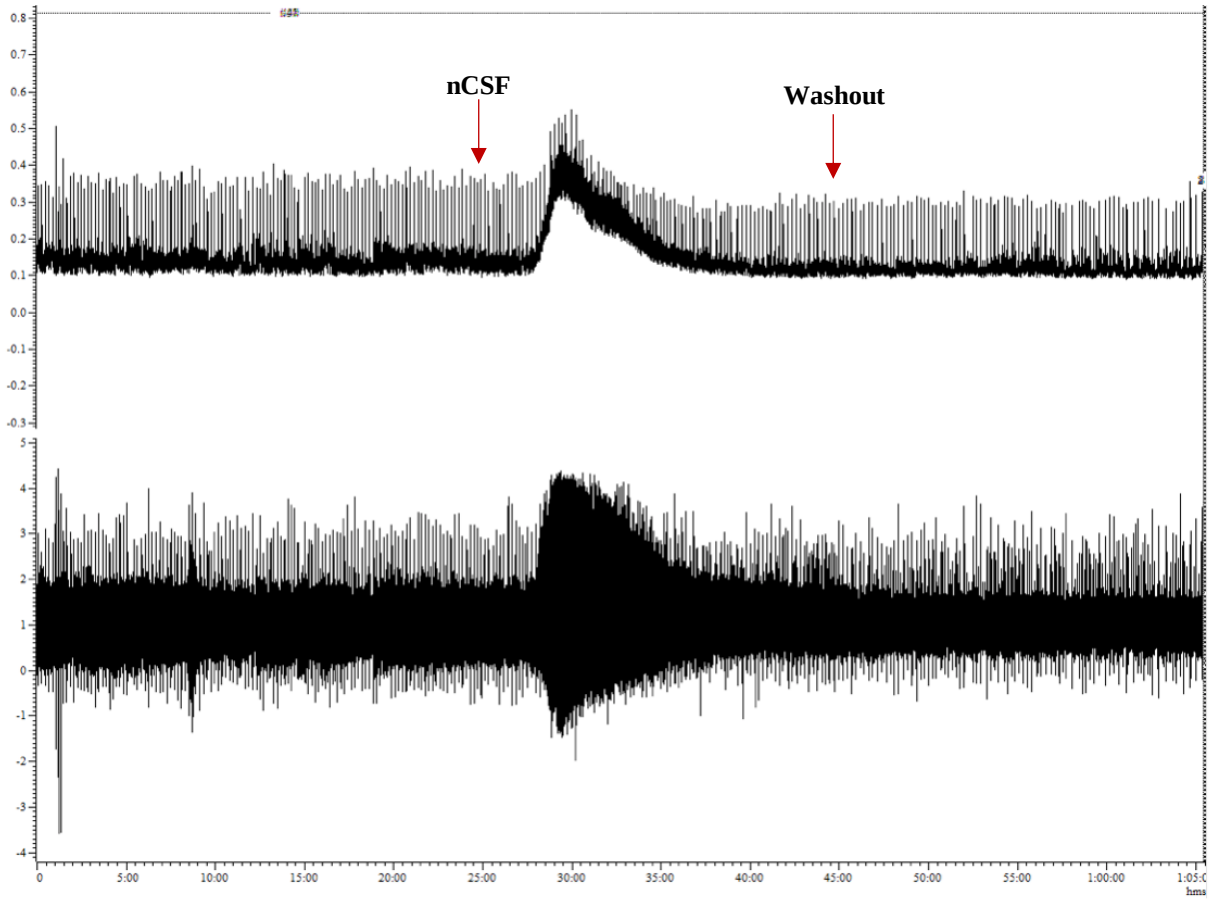

Figure 9*: Representative trace of raw record (bottom) and the rectified and smoothed record (Top) used for analysis. Arrows indicate the start and end of perfusion of high dose nicotinic aCSF (40μM) into the spinal cord chamber of the split bath.*

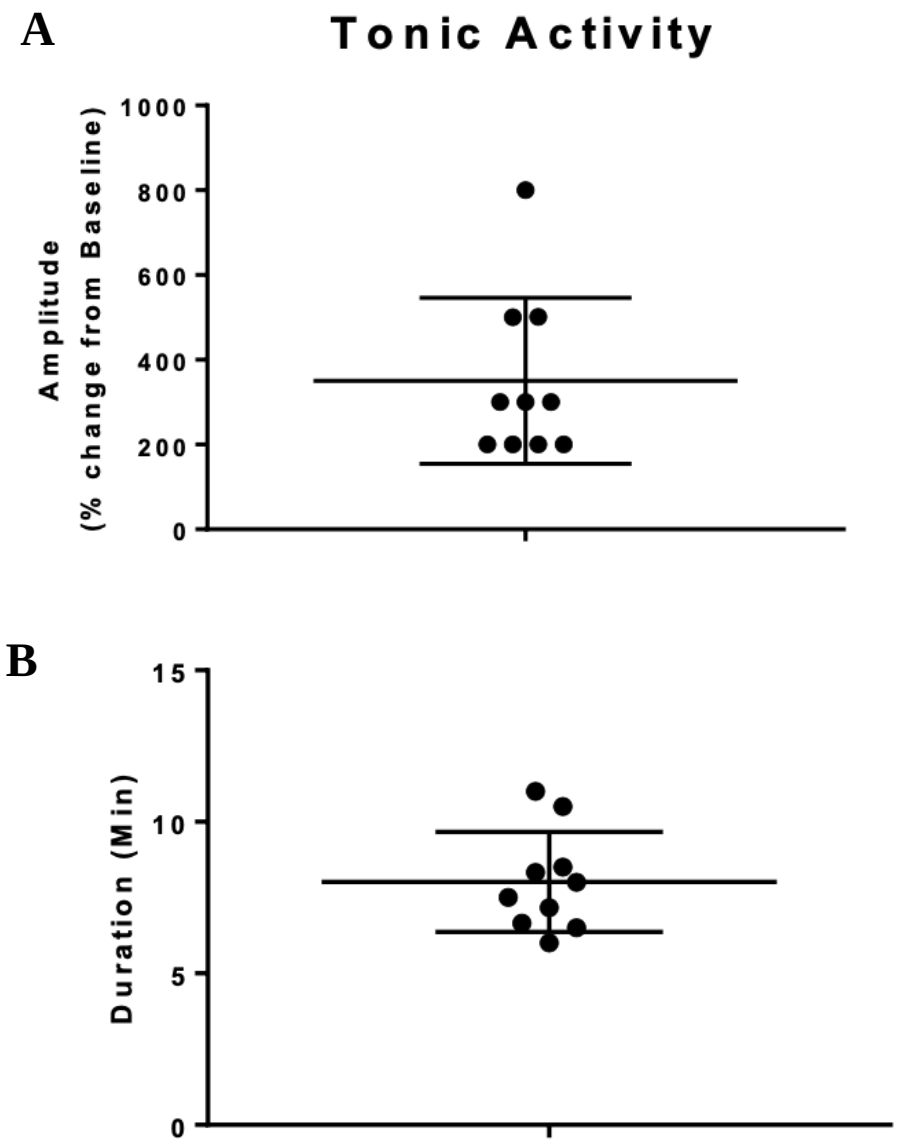

***Figure 10**: Percentage change of tonic activity caused by application of 40μM nCSF to the spinal cord perfusion chamber (Panel A). The duration of tonic activity is presented in Panel B.*

Discussion

The main findings of these experiments showed that nicotine does affect respiratory burst activity when applied to the spinal cord. Additionally, we found evidence that nAChRs desensitize after activation and these effects are highly dependent on nicotine dosage. Below, the significance of these findings and future directions are discussed.

Nicotine application alters respiratory burst amplitude. The increases in amplitude observed in these experiments, specifically those observed in the 4μM and 40μM, indicate that nAChRs are likely present somewhere along or near the phrenic motor neurons. Additionally, there was a clear dose response to nicotine application, the higher the dose the greater the increase in burst amplitude from baseline. These results indicate that nAChRs are present either on the phrenic motor neurons or on glutamatergic neurons that synapse onto phrenic motor neurons, as shown in *Figure 11*. If this were the case, activation of the nAChRs on glutamatergic neurons would trigger a release of the excitatory neurotransmitter glutamate onto PMNs, which in turn would cause increased firing of PMNs.

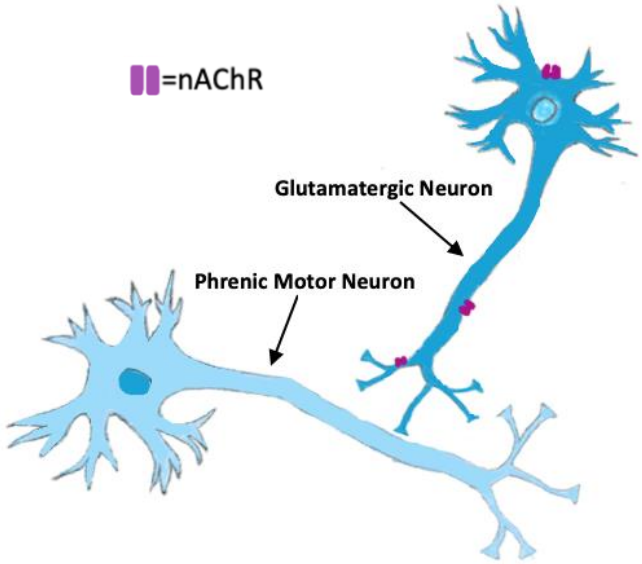

Figure 11*: Shows a Glutamatergic Neuron with nAChRs synapsing onto a Phrenic Motor Neuron. This is a probable case, if the detected nAChRs are not actually located on the Phrenic Motor Neurons themselves.*

A second observation from these experiments is that after the initial increase in burst amplitude following middle or high dose nicotine, there is a gradual decrease in amplitude. This decrease occurred despite the continued presence of nicotine in the chamber. A known characteristic of nAChRs is that they can exhibit rapid desensitization. Therefore, we hypothesize that the decrease in amplitude and area seen in these experiments is likely due to nAChR desensitization. This further indicates the presence of these receptors on or near PMNs.

Future directions. Further research can help determine whether the results of these experiments are due to activation of nAChRs on PMNs or activation of nAChRs on glutamatergic neurons that synapsed onto PMNs. The possibility of the nAChR being on glutamatergic neurons could be eliminated by blocking glutamate or by using a glutamate antagonist.

nAChRs are known to desensitize due to chronic nicotine exposure and now that we know that nAChRs are present, an important next step is to determine how they are affected by developmental nicotine exposure and whether this impairs breathing in DNE animals. Therefore, it is important to repeat these experiments using brainstem spinal-cord preparations obtained from DNE neonates to test the hypothesis that DNE causes chronic desensitization of nAChRs on or near phrenic motor neurons. If this hypothesis is correct, this chronic desensitization would be observed as a smaller increase in burst amplitude, area, and tonic activity, and potentially a

more rapid desensitization phase in response to nCSF application. These results could mean a less effective respiratory control system, whereby the animals have a harder time responding to a respiratory challenge. This could possibly translate to a contributing factor that causes SIDS.

Furthermore, the type and subtype of nAChRs can also be determined by adding type-specific antagonists to the caudal chamber during the nicotinic aCSF phase. Identifying the specific type and subtype of nAChRs on the PMNs could potentially help in the development of target treatments or drugs that could increase the receptor's desensitization threshold, help reopen desensitized receptors or lengthen activity period before they become fully desensitized.

Acknowledgements:

Thank you to everyone in the Fregosi lab for all their guidance: Emily Flanigan, Dr. Lila B. Wollman, Seres Cross, and especially Dr. Ralph F. Fregosi for allowing me to complete my honors thesis in his lab.

References

Albuquerque, E., Pereira, E.F.R., Alkondon. M., Rogers, S.W., "Mammalian Nicotinic Acetylcholine Receptors: From Structure to Function." *Physiological Reviews*, vol. 89, no. 1, 2009, pp. 73–120., doi:10.1152/physrev.00015.2008.

Jaiswal, S. J., Pilarski, J.Q., Harrison, C.M., Fregosi, R.F., "Developmental Nicotine Exposure Alters AMPA Neurotransmission in the Hypoglossal Motor Nucleus and Pre-Botzinger Complex of Neonatal Rats." *Journal of Neuroscience*, vol. 33, no. 6, 2013, pp. 2616–2625., doi:10.1523/jneurosci.3711-12.2013.

"Sudden Infant Death Syndrome and Infant Mortality." *Illinois Department of Public Health*, www.idph.state.il.us/sids/sids_factsheet.htm.

"Sudden Infant Death Syndrome (SIDS)." *Mayo Clinic*, Mayo Foundation for Medical Education and Research, 13 Nov. 2018, www.mayoclinic.org/diseases-conditions/sudden-infant-death-syndrome/symptoms-causes/syc-20352800.

Pilarski, J.Q., Wakefield, H.E., Fuglevand, A.J., Levine, R.B., Fregosi, R.F., "Increased Nicotinic Receptor Desensitization in Hypoglossal Motor Neurons Following Chronic Developmental Nicotine Exposure." *Journal of Neurophysiology*, American Physiological Society, Jan. 2012, www.ncbi.nlm.nih.gov/pmc/articles/PMC3349681/.

Shao, X.M., Feldman. J.L., "Central Cholinergic Regulation of Respiration: Nicotinic Receptors." *Acta Pharmacologica Sinica*, Nature Publishing Group, June 2009, www.ncbi.nlm.nih.gov/pmc/articles/PMC4002383/.

Wollman, L.B., Haggerty, J., Pilarski, J.Q., Levine, R.B., Fregosi, R.F., "Developmental Nicotine Exposure Alters Cholinergic Control of Respiratory Frequency in Neonatal Rats." *Developmental Neurobiology*, vol. 76, no. 10, 2016, pp. 1138–1149., doi:10.1002/dneu.22380.