THE EFFECTS OF DEVELOPMENTAL NICOTINE EXPOSURE ON MUSCLE FATIGABILITY IN NEONATAL RATS

By

MIKAYLA JANE WHALEN

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Approved by:

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Dr. Ralph Fregosi
Department of Physiology

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Abstract

This study aimed to explore the effects of developmental nicotine exposure (DNE) on the fatigability of the diaphragm in neonatal rats ages postnatal day 0-14. We hypothesized that chronic perinatal nicotine exposure would desensitize or functionally alter nicotinic acetylcholine receptors (nAChRs) at the neuromuscular junction. Muscle fatigue can be defined as the point where the muscle, in this case the diaphragm, is no longer able to produce a target force. Muscles were fatigued either through direct stimulation of the muscle or through electrical transmission through the phrenic nerve. Fatigue was quantified as the percent change in force outputs after five minutes of intermittent stimulation (330 ms trains, 0.5 trains/sec, 40 Hz, 0.2 ms pulse width). Neuromuscular transmission failure, wherein transmission across the neuromuscular junction falters, was also measured by stimulation of the phrenic nerve, as above, but with superimposed direct muscle stimulation every two seconds[9]. The results showed that there was no significant difference in muscle fatigability or the integrity of the neuromuscular junction between control and DNE animals. These results may be explained by the high safety factor present at the neuromuscular junction.

Introduction

When a muscle, including the diaphragm, is stimulated to contract the fatigue process begins. Once fatigue has begun, it does not mean contraction has failed, but that only submaximal contractions are possible until recovery can occur[2]. Developmental nicotine exposure (DNE) is suspected to have an influence on how this process occurs and perhaps interferes with physiological mechanisms to lead to early or more rapid muscle fatigue. Our hypothesis is that DNE will alter the development of post-synaptic receptors on muscle fibers,

specifically nicotinic acetylcholine receptors (nAChRs). Thus determining the difference between fatigability in DNE and control groups will give further insight as to how these receptors are affected by DNE.

Fatigue can be central or peripheral and the cause for each varies. Central fatigue occurs when motoneurons cannot be excited. Peripheral fatigue implies a failure of the neural signal to be transmitted to the muscle and thus a diminished response to the signal is observed[9]. Peripheral fatigue can be attributed to both pre and post-synaptic mechanisms. Pre-synaptic failure can be caused by a block in branch point conduction, failure of excitation-contraction coupling, reduced release of acetylcholine, or reduced quantal size. Post-synaptic mechanisms of fatigue include desensitization of cholinergic receptors, or reduced excitability of the sarcolemma[9].

Understanding the causes behind muscle fatigability leads back to the action potential. The action potential initiates contractions by activating the muscle fibers, but is greatly impacted by extracellular ion concentrations which is another factor related to fatigue. As the signal propagates through the muscle via transverse (T)-tubules, which are invaginations of the plasma membrane, voltage gated channels are able to sense the change in membrane potential which leads to the release of Ca2+ from the sarcoplasmic reticulum. Presynaptically, repetitive stimulation and exposure to high extracellular Ca2+ causes less ACh vesicles to be released leading to muscle fatigue. The repetitive stimulation also may lead to increased accumulation of extracellular K+ which inhibits repolarization and can lead to prolonged hyperpolarization[1].

The diaphragm is an interesting model for fatigue as it directly impacts respiration which requires constant contraction throughout the lifetime. The muscle itself spans the width of the ribcage and is innervated by a cervical spinal nerve, the phrenic nerve. The phrenic nerve

innervates the diaphragm which is responsible for ventilation as well as non-ventilatory functions such as coughing and sneezing.

Fatigability has been explored in relation to many other factors of human physiology, however the connection between DNE and the fatigability of the diaphragm has yet to be explored.

Methods

Beginning with dissection, week 1 rats (0-7 days) were placed in an ice bucket for several minutes for anesthetization and week 2 rats (8-14 days) were exposed to isoflurane gas for several minutes until they were non-responsive to a needle stick. Isoflurane has been shown to have no effects on the functioning of the diaphragm under fatigued and unfatigued conditions and so, should not alter measures of fatigability[6].

Rats of all ages were decapitated after they were determined to be non-responsive. Gross dissection to remove the ribcage was followed by removal of connective tissues and blood vessels to isolate the diaphragm and the phrenic nerves. Dissection was done in fresh ice-cold Krebs's solution, which was replaced at frequent intervals. Finally, the ribcage was separated from the spinal cord on both sides to create two hemi diaphragms.

During experimentation the preparation was immersed in Krebs solution in a chamber (Fig. 1) heated to 37 degrees Celsius and gassed with 95% oxygen/5% CO2. The Krebs solution was continuously exchanged via gravity perfusion and a vacuum. One end of the hemi diaphragm was pinned to the floor of the perfusion chamber, and the other end was clamped to a force transducer. With stimulation, the muscle fibers shortened and pulled on the transducer, producing a force recording that we calibrated in grams.

For muscle-only experimentation, the tension was adjusted until a twitch showed maximum force output, at which point continuous stimulation of the muscle began (330 ms trains, 0.5 trains/sec, 40 Hz, 0.2 ms pulse width). The muscle was stimulated for five minutes then single muscle twitches were done at one, two and five minutes, post-stimulation.

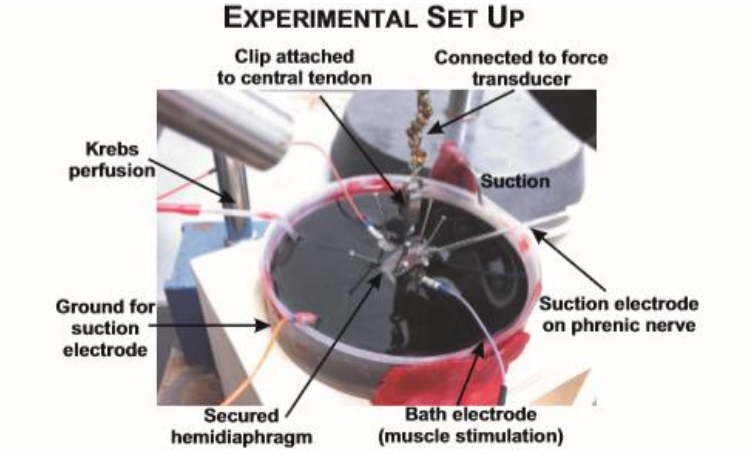


Figure 1. The experimental set up used for all three experiments.

For nerve experiments, the process was identical, however the clamp was placed so that the nerve was free from the clamp. This was done so the glass suction electrode could easily access the nerve for stimulation. At this point, if a single twitch force revealed that the nerve was able to successfully transmit a signal to the muscle resulting in a force output, the fatigue protocol began. If the stimulation of the nerve did not result in an output by the muscle, adjustments were made to pull more of the nerve into the electrode, or remove the damaged end of the nerve and a single twitch was tested again. At the end of the five-minute fatigue protocol, twitch forces were measured at one, two, and five minutes.

Neurotransmission failure experimentation began with the muscle being clamped and the nerve being pulled into the suction electrode as is described above for muscle and nerve only experiments. Once it was determined that the nerve could successfully stimulate the muscle,

intermittent train stimulation began. At fifteen second intervals, right before another nerve stimulation took place, the muscle was directly stimulated to contract, overriding the nerve stimulation. This process was continued for five minutes, at which time twitch forces were measured at one, two, and five minutes after the fatigue protocol ended. An example of one of these recordings can be found in Figure 4.

Each recording was analyzed to determine initial and final force outputs in grams. Peak force was subtracted from baseline force for the first and last force output measurements and these values were named nerve initial force, nerve final force, muscle initial force, and muscle final force. These values were then used to calculate the total change by subtracting final from initial and dividing by initial. For nerve and muscle only experiments these changes were multiplied by 100 to be measured as a percentage of the maximum force.

Neuromuscular transmission failure, referring to the inability of a neural signal to be transmitted across the neuromuscular junction, was measured using the amount of change in the values found above. These values were termed percent nerve failure (NF) and percent muscle failure (MF). Then neurotransmission failure was calculated using the equation: NTF= (NF-MF)/(1-MF). In words, if nerve stimulation causes a much greater force loss than direct muscle stimulation, then neuromuscular transmission failure is high.

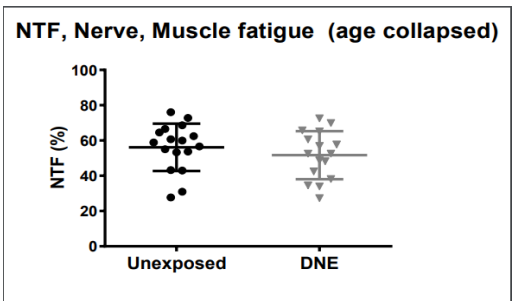
Single twitch analysis assessed the contractile properties of the muscle. We measured the time to peak force (ms), peak force in grams and ½ relaxation time (ms) to determine differences between control and DNE groups. Time to peak was measured by subtracting the time at peak from the time where force began to rise from baseline. Peak force was determined by subtracting peak force from baseline force. ½ relaxation time was measured by finding the time at which ½

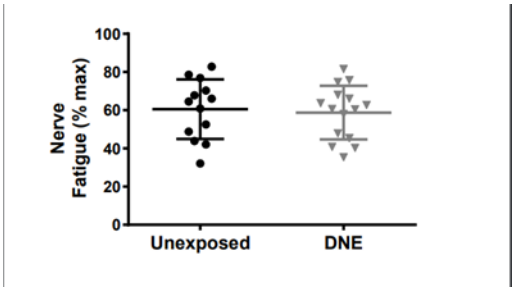
peak force intersected with the output curve. This time was subtracted from time at peak and divided by two to obtain the final value for ½ relaxation time.

Results

The results showed no significant difference between the control group and the DNE group for any of the experimental categories. Results for neurotransmission failure experiments can be found in Figure 2A. Results for nerve only fatigue are shown in Figure 2B and fatigue is measured as a percentage of the maximum force output. The results were consistent with data from previous studies that used a similar stimulation protocol[4]. Figures 3 and 4 are provided as examples of the force output throughout either the muscle only stimulation protocol (Fig. 3), or the NTF protocol, where nerve stimulation is periodically interrupted by direct muscle stimulation (Fig. 4). Analysis of these force recordings were used to create Figure 2.

Contractile properties of muscle measured by single supramaximal simulation provided measures of twitch force, contraction time and ½ relaxation time. As shown by comparing the values in Tables 1 and 2, controls had a trend towards a higher average peak force than the DNE animals, though the difference was not quite significant ($p=0.07$). DNE animals, on average, showed a trend towards a longer time to peak, but a faster ½ relaxation time. However, these trends were not significantly different, so conclusions about the impact of DNE on muscle contractile properties cannot be made.





2A. 2B.

Figure 2. A) NTF, calculated as described above, and expressed as a percentage. There is no significant difference between DNE and control groups. B) For nerve stimulation only experiments, the decline in force over the five minute stimulation protocol also showed no significant difference between groups.

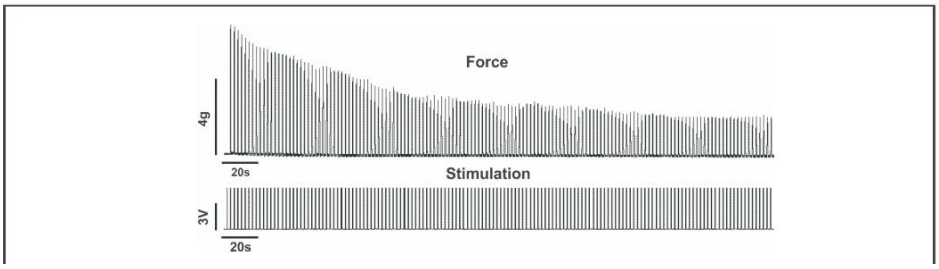


Figure 3. Measurement of muscle fatigue across five minutes of intermittent stimulation, as described above.

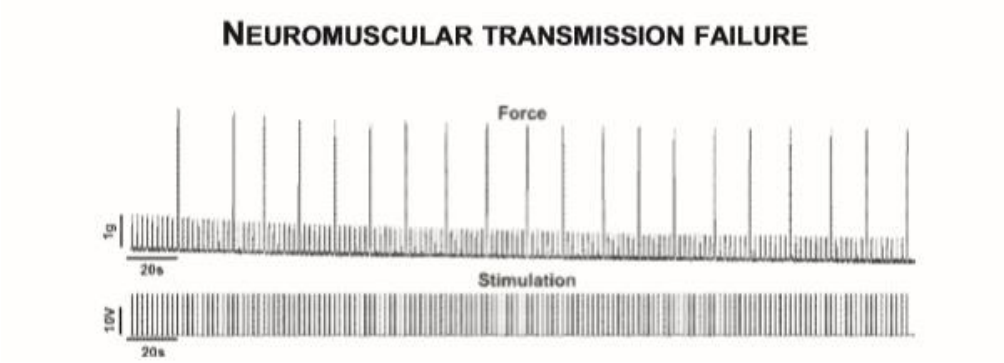


Figure 4. Neuromuscular transmission failure over a span of five minutes with continuous stimulation and an overriding muscle contraction every 15 seconds.

Twitch Characteristics (Control)

Date	Age	Time to Peak (ms)	Peak Force (g)	1/2 Relaxation Time (ms)
11.27	1	0.0785	0.438	0.02605
1.23	1	0.173	0.877	0.123
12.5	2	0.124	1.022	0.0885
1.3	2	0.0674	0.692	0.0369
11.1	3	0.1315	3.16	0.07745
11.29	3	0.183	0.9024	0.16905
12.6	3	0.091	1.2331	0.0515
12.6	3	0.096	1.516	0.05115
11.13	8	0.524	2.03	0.324
1.22	8	0.2622	1.787	0.2433
11.15	10	0.134	3.886	0.066
Averages		0.169509	1.594864	0.114264
Standard Deviation		0.130193	1.074189	0.094739

Table 1. Muscle twitch characteristics for control experiments across all ages.

Twitch Characteristics (DNE)

Date	Age	Time to Peak (ms)	Peak Force (g)	1/2 Relaxation Time (ms)
1.29	1	0.185	0.21	0.046
1.29	1	0.0813	0.408	0.03895
2.12	1	0.084	0.156	0.055
12.5	2	0.107	1.4387	0.0595
2.13	2	0.284	0.285	0.0835
11.8	3	0.473	0.496	0.2505
11.15	3	0.3048	2.24	0.1438
12.6	3	0.096	1.516	0.05115
2.5	8	0.859	0.19	0.027
2.6	9	0.0794	1.365	0.03905
Averages		0.25535	0.83047	0.079445
Standard Deviation		0.248996	0.741648	0.068601

Table 2. Muscle twitch characteristics for DNE experiments across all ages.

Discussion

Based on the results of these experiments, DNE has no effect on diaphragm fatigability and does not alter neuromuscular transmission. It was important to explore this relationship as past studies have determined that DNE has an effect on nicotinic acetylcholine receptors in the brain and it is suggested that it leads to loss of sensitivity and deactivation of these receptors[4, 7, 8]. Thus, we hypothesized that these changes would also be found in the nAChRs at the diaphragm neuromuscular junction, but the results do not support the hypothesis.

Factors to consider when measuring fatigue include age, gender, dissection time, muscle composition, muscle mass, and oxygen carrying capacity. Because muscle mass and oxygen carrying capacities differ largely across ages, age was a controlled factor, and rats were separated into week one (P0-P6) and week two (P7-P14) for the purpose of analysis. Dissection time and technique is extremely important especially when using the phrenic nerve to conduct the electrical impulse to the muscle. Long dissection times or poor technique mean too much exposure to air, not enough oxygenation, and ultimately death of the nerve and later the tissue. Thus, it is important to keep dissection fast and consistent to accurately interpret results.

Although the results show no difference in the muscle fatigue of these two groups this is an important conclusion to make and brings us closer to fully understanding the effects of DNE. The "safety factor" at the neuromuscular junction is likely a large reason for why there is no difference between the groups. In an effort to protect desensitized receptors, an excess of neurotransmitter, in this case acetylcholine, is released at the neuromuscular junction, and as a result the same effect is produced at both normal and desensitized receptors. Future studies should conduct more experiments to create a more comprehensive data pool which better represents all ages, P0-P14. Additionally, studying various temperatures and nicotine

concentrations could be interesting directions to potentially identify the differences between control and DNE animals.

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