EFFECT OF MICROGRAVITY ON THE ACTIVE ZONES OF THE NEUROMUSCULAR SYSTEM

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Abstract

It is known that periods of unloading lead to muscle atrophy and disruption of neuromuscular function. To explore how microgravity would affect the neuromuscular junction, the active zones (Bassoon and P/Q calcium channels) of rat soleus, a slow-twitch, weight-bearing muscle, and plantaris, a fast-twitch, non-weight-bearing muscle, were examined. Post-synaptic Acetylcholine receptors, along with pre-synaptic P/Q calcium channels and Bassoon protein were stained and measured. Results showed that there was only a significant change in plantaris active zones, while there was nothing of significance in the soleus. Specifically, there was a significant change in P/Q and Bassoon/Acetylcholine area in all the unloaded rats. Male unloaded rats also showed a significant change in both Bassoon and Acetylcholine receptors. Looking at values that were trending towards significance, a majority were also males, allowing us to explore the possibility that males are more susceptible to neuromuscular decay. Moreover, as rats are still able to stretch while in hindlimb suspension, they are still able to use their soleus muscles but are not recruiting their plantaris muscles. Therefore, astronauts, especially men, that will experience microgravity for any length of time should engage in intense strength and endurance training to recruit those fast twitch muscles.

Introduction

Outer space remains one of the final frontiers for human exploration. In order to gather knowledge about other planetary bodies, it is imperative to understand how foreign conditions of microgravity affect human bodies. One large remaining concern regarding space travel is the effect microgravity has on the neuromuscular system, which is responsible for locomotion and other mission specific tasks. It is understood that leaving Earth's atmosphere for any length of time will lead to a decrease in muscle mass as well as strength (Ikemoto et al., 2001). Even a 14-day spaceflight was found to be more detrimental to muscles than 20 days of bedrest (Akima et al., 2001). Significant muscle atrophy results from small increments of spaceflight, but as space exploration expands further, so will the length of time that astronauts will be exposed to microgravity (LeBlanc, Rowe, Schneider, Evans, & Hedrick 1995). In order to combat this muscle atrophy, the mechanisms behind it must be understood. (Ikemoto et al., 2001).

Even though extensive research regarding neuromuscular junctions (NMJs) has been done, those barely scratch the surface; more research needs to be conducted. In a 2009 study, it was found that women were more affected by muscle unloading than men (Deschenes, McCoy, Holdren, & Eason). These differences in reduced strength were said to be "associated with similar disparities in the nervous system's capacity to maximally stimulate muscle" (Deschenes et al., 2009). It was thought that this might be due to fatigue, however, later it was found that female muscles were actually less susceptible to fatigue (Deschenes & Leathrum, 2016). In order to explain this disparity in gender, the active zones of NMJs, where the release of neurotransmitter-containing vesicles occurs, were examined. More specifically, bassoon proteins and P/Q channels of NMJs of male

and female rats using the hindlimb suspension model to mimic conditions of microgravity, as first described by Morey et al. (1979).

P/Q channels are calcium channels found at nerve terminals that are instrumental in facilitating neurotransmitters to send signals to receptors (Ishikawa, Kaneko, Shin, & Takahashi 2005). Similarly, Bassoon is also a key protein in active zones responsible for vesicle docking in proper positions (Sudhof, 20102). Bassoon is a presynaptic protein that docks pre-synaptic vesicles to the active zone, again playing a crucial role in neurotransmission (Sudhof, 2012). We expected that non-weight bearing muscles would demonstrate resistance to unloading (and thus microgravity) as they should be relatively unaffected by the treatment (Deschenes, Wilson, & Kraemer 2005).

Experimental Procedure

For this investigation, forty young adult rats split into four groups were studied: 1) male control, 2) female control, 3) male unloading, and 4) female unloading. To mimic the conditions of microgravity experienced by astronauts, the hindlimb suspension model first described by Morey and her colleagues was implemented (1979). During the two-week intervention, control rats were kept in normal housing conditions, two rats per tub and received food and water *ad libitum* (Deschenes & Leathrum, 2016). Immediately following, the rats were anesthetized with a cocktail of ketamine and xylazine. The soleus and plantaris muscles were then extracted and their physiological function was tested. Using Aurora Scientific Inc. in vitro muscle stimulating and recording system, muscle contraction was induced through nerve terminal excitation as well as through direct muscle stimulation. The results of these trials showed significant effects for treatment and gender (Deschenes & Leathrum, 2016).

Data Collection

The remaining muscle samples were frozen at resting length and stored at -80°C. Once frozen, four 50 μm thick longitudinal slices from the middle third of each muscle were mounted on EDTA-treated slides for analysis. During a two-day staining procedure, anti-bassoon and anti-P/Q primary antibodies were administered to the muscle. After an incubation period, secondary antibodies of Alexa 647, Alexa 488, and rhodamine conjugated Bungarotoxin were added to provide immunofluorescent labelling. An Olympus confocal microscope using a TRF 1000x objective was then used to look at and image acetylcholine receptors, P/Q channels, and bassoon protein. 12-15 images were taken per slide.

These images were then analyzed using Image-Pro Plus, taking the total perimeter (length around the visible area), total stained area (sum of lengths around each separate receptor cluster), total area (all of the stained clusters and area between), and stained area. Finally, the dispersion (stained area vs total area) was calculated.

Statistical Analysis

Following the data collection outlined above, the information was evaluated using a two-way ANOVA, with the main effects of treatment (unloading) and gender for each muscle type. In the event of a significant effect, a Tukey post-hoc test was utilized to identify significant pairwise differences. In all cases, a p-value of ≤ 0.05 was used to determine significance.

Results

Looking at the results from the plantaris muscles compared to those of the soleus muscle, there is an obvious discrepancy between the two - there were no values of statistical significance in the soleus muscle. This can be seen in table 1.

		Male Control	Male Unloaded	Female Control	Female Unloaded
Mean Ach Receptors	Manual Perimeter	133.1 ± 28.8	130.4 ± 23.6	119.3 ± 18.9	134.8 ± 27.9*
	Automatic Perimeter	293.0 ± 78.2	326.8 ± 86.2	275.8 ± 43.3	288.1 ± 69.9
	Total Area	469.7 ± 117.1	503.7 ± 133.8	456.9 ± 94.7	481.1 ± 143.5
	Stained Area	187.5 ± 55.4	204.1 ± 51.9	177.5 ± 35.8	197.1 ± 55.8
	Dispersion	41.8 ± 5.5	42.6 ± 7.9	41.3 ± 3.9	43.2 ± 6.6
Mean Bassoon Receptors	Manual Perimeter	148.9 ± 87.7	117.3 ± 22.4	105.4 ± 16.4	107.8 ± 16.0
	Automatic Perimeter	621.0 ± 226.3	807.8 ± 220.0*	626.4 ± 143.2	653.6 ± 206.9
	Total Area	432.0 ± 119.9	474.8 ± 116.8	413.2 ± 83.1	460.7 ± 143.1
	Stained Area	134.2 ± 50.3	132.4 ± 51.7	110.9 ± 26.0	123.1 ± 47.8
	Dispersion	31.6 ± 10.0	27.1 ± 4.1	28.8 ± 6.6	28.4 ± 7.7
Mean PQ Channels	Manual Perimeter	133.0 ± 53.7	121.4 ± 24.3	106.1 ± 15.0	120.1 ± 34.6*
	Automatic Perimeter	402.6 ± 111.5	460.1 ± 149.2	377.5 ± 102.4	442.0 ± 137.1
	Total Area	498.6 ± 131.2	583.8 ± 181.1	483.7 ± 108.9	533.3 ± 153.1
	Stained Area	227.1 ± 57.6	277.5 ± 92.9*	226.0 ± 73.5	240.8 ± 72.7
	Dispersion	47.0 ± 9.4	42.9 ± 17.2	47.6 ± 9.8	45.5 ± 4.3

Table 1. Neuromuscular junction active zones in soleus muscle of female and male rats after 2 wk experimental period.
Values are means ± standard deviation, N=10/group.
*indicates a large Cohen's d effect size for treatment (d > 0.8)

		Male Control	Male Unloaded	Female Control	Female Unloaded
Mean Ach Receptors	Manual Perimeter	98.5 ± 18.9	85.2 ± 9.9*	93.2 ± 10.0	89.6 ± 7.5
	Automatic Perimeter	250.7 ± 82.1	190.5 ± 27.8**	225.4 ± 56.1	200.7 ± 39.8
	Total Area	368.4 ± 95.0	311.3 ± 61.1*	355.9 ± 50.0	312.1 ± 61.4*
	Dispersion	42.5 ± 9.8	45.3 ± 8.2	47.7 ± 5.9	45.3 ± 9.2
Mean Bassoon Receptors	Manual Perimeter	88.0 ± 14.3	77.5 ± 5.5*	84.4 ± 7.8	82.4 ± 6.2
	Automatic Perimeter	628.3 ± 112.0	511.5 ± 144.9	608.3 ± 131.7	591.4 ± 80.7
	Total Area	365.1 ± 91.0	310.1 ± 49.9	345.6 ± 70.6	322.3 ± 45.7
	Dispersion	28.5 ± 4.4	40.5 ± 8.0**	35.6 ± 9.4	36.7 ± 4.2
Mean PQ Channels	Manual Perimeter	92.4 ± 17.5	79.7 ± 6.6*	86.5 ± 8.5	84.0 ± 6.8
	Automatic Perimeter	329.2 ± 90.2	236.2 ± 52.5*	285.8 ± 50.7	285.9 ± 60.6
	Total Area	393.9 ± 104.6	323.8 ± 60.9**	379.0 ± 75.3	324.2 ± 45.7**
	Dispersion	39.0 ± 7.2	46.8 ± 6.4	46.2 ± 8.3	44.2 ± 11.9
BSN/BTX	Area	74.3 ± 14.6	106.7 ± 43.3**	70.8 ± 21.9	93.8 ± 16.3**
PQ/BSN	Area	266.2 ± 195.4	142.9 ± 61.1*	186.4 ± 64.0	145.7 ± 49.7*

Table 2. Neuromuscular junction active zones in plantaris muscle of female and male rats after 2 wk experimental period.
Values are means ± standard deviation, N=7/group.
**indicates significant (P ≤ 0.05) difference between hind limb suspension and control
*indicates trend towards significance (0.10 > P > 0.05)

Upon looking at the effect size of treatment some values that showed a large effect size of greater than 0.8 were female manual perimeter of Acetylcholine receptors, male stained perimeter of Bassoon protein, female total perimeter of P/Q channels, and male stained area of P/Q channels, showing that there indeed was a moderate change in neuromuscular active zone, although it was not enough to obtain statistical significance. Perhaps if the trial were longer, we may have seen more of a difference.

In table 2 above, looking at the active zones in the plantaris muscle, we did obtain some values of statistical significance. Most notably, there was a significant change in the total area of the P/Q channels in both the unloaded male and female rats. In addition, all unloaded subjects show a significant change in the Bassoon over Acetylcholine area, showing that in relation to the Bassoon that stayed relatively constant, the area of Acetylcholine receptors started to decay. Female rats did not see any other significant

changes, but the male rats also saw a change in the automatic perimeter of Acetylcholine receptors and the dispersion of Bassoon proteins. In looking at values that showed a trend toward significance ($0.10 > P > 0.05$), many of them were found in the male unloaded rats, showing that males may be more susceptible to neuromuscular decay over a shorter period of time.

Discussion

These results have multiple important facets. The first having to do with the fact that the slow twitch, non-weight-bearing muscle, soleus, did not experience any significant amount of neuromuscular active zone decay following a two-week period of unloading. This makes sense, as the rats are able to still stretch their legs as well as point their feet, all which recruit the soleus muscle, which is an ankle extensor muscle (Armstrong & Laughlin, 1985). The plantaris muscle, which is also an ankle extensor muscle, is only recruited during intense endurance and strength training, so it is never being used by the rat while in the hindlimb suspension model (Armstrong & Laughlin, 1985). This reasoning is supported by the data that was collected – the plantaris muscle was the only muscle to show any change that was of statistical significance.

The second thing to note is that the male unloaded plantaris muscles had an increased incidence of significance as well as trends towards significance as compared to the female unloaded plantaris muscles. Previous studies have shown a disparity between male and female neuromuscular junction areas. In that study, however, it was found that females were *more* susceptible to muscle unloading (Deschenes, McCoy, & Mangis, 2012). They supposed that this was due to disturbances n neural drive from the central nervous system (Deschenes, McCoy, & Mangis, 2012). These disparities were found in EMG activity, but not force relative to EMG, neuromuscular transmission, or muscle mass (Deschenes, McCoy, & Mangis, 2012). The data collected in this study shows the opposite result looking within the neuromuscular junction. This work implies that male neuromuscular junction active zones will decay more rapidly than that of a female. In other words, female neuromuscular junction active zones are more resistant to unloading, in strict terms of neuromuscular active zones than that of males.

Finally, in looking at a similar study of the same length of hindlimb suspension, myofibers of the same muscles showed significant atrophy (Deschenes et al., 2017). Therefore, there is some difference in sensitivity to changes in activity between the neuromuscular junctions (specifically active zones) in comparison to the myofibers they innervate.

Should this study have been carried out over a longer period of time, we suspect that there would have been more significant changes in the female neuromuscular junctions of the unloaded rats, as well as some statistically significant changes in the soleus muscle. Future studies might also look at the effects of hindlimb suspension on the neuromuscular active zones of aged rats, as studies have shown that aged rats predisposed to decay as a function of their age (Deschenes, Tufts, Noronha, & Li, 2018).

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