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Cerebellar Abnormalities in Developing DMD Mdx Mice and Respiratory Compensatory Responses to Acute Isocapnic Hypoxia

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CEREBELLAR ABNORMALITIES IN DEVELOPING DMD *MDX* MICE AND RESPIRATORY COMPENSATORY RESPONSES TO ACUTE ISOCAPNIC HYPOXIA

by

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Abstract

Previous studies have analyzed causal factors among infants who succumb to Sudden Infant Death Syndrome (SIDS). Two causal factors include the presence of a brain abnormality and exogenous stressors, it was hypothesized that cerebellar Purkinje cell (PC) dysfunction would inhibit ventilation during graduated challenges of isocapnic hypoxia conditions (19%, 17%, 15%, and 13% O_2/ 3% CO_2) compared to normal room air (21% O_2, 0% CO_2, 79% N). The challenges of isocapnic hypoxia conditions were chosen to simulate an environment with exogenous stressors that an infant with an unknown brain abnormality might experience. Experiments were carried out on sibling pairs of a mouse model of Duchenne's muscular dystrophy (C57BL/10ScSn-*Dmd*^{mdx}/J). Minute ventilation, comprised of tidal volume and breath frequency, was measured using whole body plethysmography during baseline exposure to normal room air and to isocapnic hypoxia conditions between both groups at post-natal day 62 (n = 7 for both wildtype and DMD *mdx* mutant). Groups did not show a significant difference in ventilatory responses during isocapnic hypoxia conditions compared to baseline. The combination of the dual gas challenge could have the effect of both decreasing and increasing ventilation responses simultaneously. Future research should focus on a single challenge of hypoxia or hypercapnia or chronic exposure to hypoxia or hypercapnia to explore the possibility of revealing respiratory responses much differently than those exposed to acute isocapnic hypoxia.

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CHAPTER 1: INTRODUCTION

Sudden Infant Death Syndrome

Sudden Infant Death Syndrome (SIDS) is the third leading cause of infant mortality in the U.S. among infants less than one year of age (Eunice Kennedy Shriver National Institute of Child Health and Human Development, 2013). Not only is SIDS the third leading cause of infant mortality, but also it is the number one cause of infant mortality with an unknown mechanism of action (Centers for Disease Control and Prevention, 2015; Willinger, James, & Catz, 1991). Male infants are especially vulnerable to SIDS with higher mortality rates than female infants (Hoyert & Xu, 2012; National Sleep Foundation, 2015; Trachtenberg, Haas, Kinney, Stanley, & Krous, 2012). Suggested SIDS risk indicators appear as early as one week after birth in infants who later succumb to SIDS with the majority of deaths occurring between two and four months of age (Eunice Kennedy Shriver National Institute of Child Health and Human Development, 2013). These risk indicators may manifest, among other characteristics, as distortions in sleep state organization, periods of tachycardia, diminished compensatory cardiorespiratory responses to challenging environmental situations, respiratory pauses (apnea), an increased incidence of obstructive apnea, especially during sleep, and decreased spontaneous movement (Eunice Kennedy Shriver National Institute of Child Health and Human Development, 2013; Harper & Kinney, 2010).

The early 1990s discovery of the relationship between increased SIDS risk and sleeping in the prone position led to the "Back to Sleep" campaign which turned into the current "Safe to Sleep" campaign (Eunice Kennedy Shriver National Institute of Child Health and Human Development, 2015). The "Safe to Sleep" campaign aims to educate parents, care givers, and health care providers about ways to reduce the risk of SIDS including placing an infant in the

supine position for sleeping rather than prone to reduce the risk of challenging carbon dioxide (CO_2) accumulation for the infant (Eunice Kennedy Shriver National Institute of Child Health and Human Development, 2013). Since the implementation of the campaign in the mid-1990s, the rate of infant deaths attributed SIDS has decreased, but SIDS still remains a leading cause of death in infants (Eunice Kennedy Shriver National Institute of Child Health and Human Development, 2013). The decrease in SIDS mortality rate could likely be attributed to a decrease in SIDS diagnoses because of technological advances in medical testing, suggesting that environmental challenges are only a partial contributor of vulnerability in infants that fall victim to SIDS (Courts & Madea, 2010; Eunice Kennedy Shriver National Institute of Child Health and Human Development, 2013; Shapiro-Mendoza, Tomashek, Anderson, & Wingo, 2006).

The Triple Risk Model

The Triple Risk Model of SIDS, aimed to find causal factors associated with SIDS vulnerability, was developed by Filiano and Kinney in 1994 and later endorsed by the National Institutes of Health (NIH, 2014). The 1994 Filiano and Kinney SIDS risk model identified three components that must converge for an infant to fall victim to SIDS: an unknown brain abnormality, a critical developmental period in homeostatic control, and presence of an exogenous stressor. Initially, an infant must have an unknown brain abnormality to be included in the Triple Risk Model.

Unknown Brain Abnormalities

A key component of the SIDS Triple Risk Model is the presence of an unknown brain abnormality (Filiano & Kinney, 1994). Extensive research has been performed on possible abnormalities within the brainstem because control of the autonomic cardiorespiratory system lies within this structure (Purves et al., 2011). However, research has yet to establish a causal

link between the brainstem and SIDS susceptibility; thus the focus has recently shifted from the brainstem to other regions that may modulate respiratory control such as the hippocampus, the serotonergic system, and the cerebellum (Cruz-Sanchez et al., 1997; Kinney et al., 2014). A unique area of investigation into causal factors for SIDS in the central nervous system (CNS) includes serotonin (5-hydroxytryptamine; 5-HT) neurons, which modulate and integrate many different compensatory functions including gasping and ventilation, thermoregulation, autonomic control, responses to changes in CO_2 and oxygen (O_2), and arousal from sleep (Corcoran et al., 2014). Compensatory responses in challenging environmental situations are critical because dysfunctions contribute to increased CO_2 accumulation, decreased levels of O_2, hypo- or hyperthermia and could prove to be fatal (Corcoran et al., 2014). Further evidence from studies analyzing 5-HT dysfunction in human and animal models that some of these 5-HT dependent compensatory functions within the CNS have diminished responses to stressors that occur during sleep, which may contribute to SIDS vulnerability in infants (Courts & Madea, 2010; Hilaire et al., 2010; Hodges & Richerson, 2010; Kinney et al., 2014; Paterson et al., 2006).

Other brain areas that are more recently being investigated include the cerebellum and its role in compensatory cardiorespiratory control. Specifically, 100% developmental cerebellar Purkinje cell loss in a mouse has been shown to reduce the response to and recovery from CO_2 compared to mice with normal cerebellar Purkinje cells (Calton, Dickson, Harper, Goldowitz, & Mittleman, 2014). Interestingly, cerebellar Purkinje cell loss is the most consistent finding in children with autism spectrum disorders (ASD; Fatemi et al., 2012). While ASD and Duchenne's muscular dystrophy (DMD) have not been systematically compared with sleep disordered breathing, both disorders show similar symptomology, especially cerebellar dysfunction, that can lead to disordered breathing during sleep (Fatemi et al., 2012; NIH, 2015; Purves et al., 2011).

Research has shown a significant association between the prevalence of DMD and autism spectrum disorder (Wu, Kuban, Allred, Shapiro, & Darras, 2005). DMD is histologically similar to ASD because both disorders display a dysfunction of cerebellar Purkinje cells and disruption in the CNS (Anderson et al., 2002; Purves et al., 2011). Sleep disordered breathing in humans diagnosed with DMD begins in the early teenage years, while breathing abnormalities attributed to lung capacity begin to decrease around 10 years of age (Simonds, 2002). While disordered breathing is typically not seen until later in life in humans diagnosed with DMD it remains to be examined whether animal models of DMD could show significant disordered breathing due to brain abnormalities in earlier stages of development before the onset of diaphragmatic dystrophy. Previous research on the convergence of two SIDS risk factors, along with cerebellar Purkinje cell loss or disruption during infancy, seems to support the hypothesis of a critical age of vulnerability when exogenous stressors can negatively influence the development of the central nervous system (Kinney et al., 2009; Simonds, 2002; Stiles & Jernigan, 2010).

Critical period of vulnerability

Along with an unknown brain abnormality, the Triple Risk Model accepted by NIH states that SIDS victims are experiencing a critical period of vulnerability (Filiano & Kinney, 1994). As mentioned before, infants are most susceptible to SIDS between one week after birth and before one year of age; a known critical period of brain development (Stiles & Jernigan, 2010). The majority of SIDS deaths occur before an infant reaches six months old, with peak susceptibility around 90 days of age (Trachtenberg, Haas, Kinney, Stanley, & Krous, 2012). The brain of a postnatal infant grows incredibly fast during the first year of birth. This is the period of time where the infant develops compensatory responses to the environment with more controlled behaviors such as target-directed hand-eye coordination and reaching to grasp, while hand-to-

hand transfer coordination occurs around six months of age (Rothbart, Posner, & Boylan, 1990). The development of these motor skills, centered in the cerebellum, coincides with the increased growth activity of the cerebellar cortex (Volpe, 2009). Because of the complexities of infant brain development this vulnerable period of rapid growth could cause the infant to be more susceptible to exogenous stressors.

Exogenous stressors

According to the Triple Risk Model detailed by Filiano and Kinney the last overlapping factor that contributes to SIDS is that an infant was exposed to one or more exogenous stressors (1994). Some documented exogenous stressors that may contribute to SIDS include being placed or found in a prone/side sleep position, race, gender, prenatal maternal alcohol intake, and postnatal maternal alcohol intake while breastfeeding the baby (Eunice Kennedy Shriver National Institute of Child Health and Human Development, 2013; Trachtenberg et al., 2012). Other documented stressors that may contribute to SIDS and result in challenging levels of CO_2 in the infants environment include the head being covered, sleeping on an adult mattress, couch, or playpen, soft bedding, and bed sharing (Eunice Kennedy Shriver National Institute of Child Health and Human Development, 2013). In one study of exogenous stressors of sudden infant death, 85% of over 200 deaths attributed to SIDS were associated with exogenous stressors that could also lead to asphyxia (Pasquale-Styles, Tackitt, & Schmidt, 2007). Maternal smoking prenatally and smoke exposure postnatal are also considered to be a possible exogenous stressor of SIDS as they decrease the level of available oxygen in the environment (Fleming & Blair, 2007). Exposure to exogenous stressors in at-risk infants may have an additional negative impact on the development of the infant brain that leads to brain dysfunction during the critical age of vulnerability. The two combined dysfunctions could negatively impact the appropriate

cardiorespiratory responses to increased CO_2 (hypercapnia) or decreased O_2 (hypoxia) or both (Kinney et al., 2009).

Normal and Challenged Respiratory Responses

A normal breath consists of multiple components including tidal volume (TV) and breath frequency (*f*), which comprise minute ventilation (TV; ml) (Walker, Hall, & Hurst, 1990). The tidal volume is the volume of normal air in a breath (Blahd, 2014). Breath frequency is the total number of breaths taken in 1 min - typically 30 to 60 breaths per minute in infants less than six months old without cerebellar abnormalities (Blahd, 2014; Walker, Hall, & Hurst, 1990). Environmental respiratory challenges that an infant may encounter include hypercapnia and hypoxia (Michiels, 2004). The normal response to both hypercapnia and hypoxia is to increase minute ventilation either by increasing TV or *f* or both (Michiels, 2004; Wakai, Takamura, Morinaga, Nakamuta, & Yamamoto, 2015).

Typical hypoxia symptoms in humans include rapid breathing, shortness of breath, and a faster heart rate (Seidu, 2014). The levels of increased ventilation and cardiac output increase the O_2 levels within the blood during hypoxic conditions (Micvhiels, 2004). The demand for O_2 is positively correlated to cerebral blood flow and the longer the duration of hypoxic conditions the more likely the brainstem, hippocampus, and the cerebral cortex will be negatively affected (Ahearne, Bocylan, & Murray, 2016; Michiels, 2004). A decrease in O_2 concentration within the blood flow of the cerebral cortex can cause brain injury such as hypoxic-ischaemic encephalopathy, which can affect feeding, irritability, reflexes, and cause seizures (Huang & Castillo, 2008).

Hypercapnia is defined as an abnormal elevation of carbon dioxide within the blood stream. Hypercapnia shares many similar symptoms as hypoxia such as, raised blood pressure,

faster breathing, as well as headaches and possible confusion (Tintinalli & Stapczynski, 2015). Hypoxia and hypercapnia can present concurrently in infants during a variety of situations, such as when infants sleep on their stomachs or have their faces turned into soft bedding, smoking in the presence of infants, or in infants with a concurrent respiratory disease (Cheng, Albanese, Ursino, & Chbat, 2016).

In addition to the individual hypoxic and hypercapnic challenges, the dual challenges of isocapnic hypoxia have also been analyzed. Isocapnic hypoxia maintains a low level of CO_2 during respiratory challenges while simultaneously decreasing O_2 in the environment (Slingo, Turner, Christian, Buckler, & Robbins, 2014). A more recent study performed in a mouse model of Chuvash polycythemia, an autosomal recessive disorder in humans that leads to hypoxic sensitivity, revealed a significantly higher respiratory response to the isocapnic hypoxia condition with a 3% increase in carbon dioxide levels compared to the hypoxia and normal room air conditions only (Slingo, Turner, Christian, Buckler, & Robbins, 2014). Further, isocapnic hypoxia conditions are representative of a situation when an infant is both O_2 deprived as well as exposed to an increase in CO_2 levels (e.g., when an infant is lying prone and someone is smoking in close vicinity of the infant). Research examining isocapnic hypoxia ventilation has commonly used a 3% increase in CO_2 levels within the experimental condition (Cheng et al., 2016; Slingo et al, 2014). The first year of birth is a particularly sensitive time when the brain is at a critical stage of developing compensatory respiratory responses to these challenging conditions (Fredericks & Saladin, 1996; Holland et al., 2014).

Cerebellar Involvement in Breathing.

The brain of a postnatal infant grows incredibly fast during the first year of birth. The cerebellum grows at the highest rate and can more than double in the first three months of age,

increasing in volume by 88% over the first year. Thus, this becomes a critical age of vulnerability where injury or dysfunction of this structure can subsequently lead to challenged breathing abnormalities (Fredericks & Saladin, 1996; Holland et al., 2014; Tau & Peterson, 2010). One of the earliest findings in relation to the cerebellum and ventilation by Mansfeld and Tyukody in 1936 observed an unanesthetized cerebellectomized dog which exhibited depressed ventilatory responses to inhalation of 10% and 20% carbon dioxide in addition to several levels of hypoxia. Further studies have found that cerebellectomy significantly attenuated minute ventilation during a response to reduced O_2 and increased CO_2 in a dog model as well as a cat model, but did not have a significant respiratory alteration on normal, at rest breathing during normal atmospheric conditions (Senapati et al., 1990; Xu & Frazier, 2000; Xu, Owen, & Frazier, 1995). Ablation of one pair of cerebellar nuclei, the fastigial nuclei (FN), produced respiratory dysfunction in cats including gasping and reduced breath frequency in response to hypoxic challenges (Xu et al, 1995). Breath frequency in infants with cerebellar abnormalities with Purkinje cell loss was reduced compared to infants without cerebellar abnormalities and former infants exhibited increased signs of apnea (Haddad, Abman, & Cernick, 2002).

The cerebellar Purkinje cells are the sole output of the cerebellar cortex (Purves et al., 2011). These Purkinje cells project through the deep cerebellar nuclei that are essential for respiratory responses, specifically the FN (Purves et al., 2011; Xu & Frazier, 2002). The other two nuclei of the cerebellum, the interposed and lateral nuclei (IN, LCN) are responsible for pulmonary mechanoreceptor-mediated respiratory responses whereas the rostral fastigial nuclei (FNr) have been shown to be essential specifically in facilitating respiratory responses to changes in CO_2 and O_2 (Xu & Frazier, 2002). The *Lurcher* mouse with nearly 100% Purkinje cell loss within the cerebellar cortex, showed significantly reduced respiratory compensatory

responses to increasing hypercapnic conditions and to a less significant degree to hypoxic conditions (Calton et al., 2014).

Duchenne's Muscular Dystrophy

Duchenne's muscular dystrophy is an X-linked recessive degenerative neuromuscular disorder that is classified by a reduction of dystrophin protein in the muscle as well as normally present in the CNS, especially the cerebellum (Holland et al., 2014). ASD and DMD have many overlapping symptoms such as motor skills deficits, decreased respiratory function, reduced attention focusing, as well as verbal learning and memory impairments (Fatemi et al., 2012; NIH, 2015). In physiological studies, these comorbid symptoms are more severe in ASD than in DMD (Fatemi et al., 2012). DMD is found in 1 of 3,600 boys (Bushby, 2012; NIH, 2015) compared to ASD, which is diagnosed in 1 in 42 boys (Bao, 2014). In humans, one-third of boys with DMD exhibit non-progressive synaptic impairment compared to boys of a similar age (Pane et al., 2012; Wu et al. 2005). The lack of dystrophin in the fetal brain may lead to the disruption of synapse formation and subsequent synaptic function in the Purkinje cells of the cerebellum (Hinton & Cyrulnika, 2004). Cerebellar Purkinje cells are particularly sensitive to abnormalities in protein expression (e.g., reduced dystrophin, essential in normal muscle function and synaptic expression; NIH, 2015; Ohlendieck et al., 1993). Specifically, a 90% decrease in dystrophin related protein expression results in decreased cerebellar Purkinje cell size, and a decrease in cellular pathway signaling (Fatemi et al., 2012; NIH, 2015; Ohlendieck et al., 1993). Immunohistochemical studies of normal mice brains have demonstrated that dystrophin is located in the cerebral cortex, the hippocampus, and the cerebellum (Hinton & Cyrulnika, 2004; Lederfein et al., 1992; Lidov, Byers, Watkins, & Kunkel, 1990; Vaillend, Billard, & Laroche, 2004). Similar to humans who suffer from DMD, one animal model of DMD, the *mdx* mutant

mouse, does not express dystrophin and therefore is routinely used as an animal model of the disease even though the myopathology is much less severe compared to the human disease course (Jackson Laboratory, 2014). Accordingly, the DMD *mdx* mouse model is considered a better model than other DMD mouse strains for examining CNS impairment related to dystrophin depletion rather than the musculature deficiencies of the disease (Beastrom, Lu, Macke, Canan, Johnson, Penton, & Montanaro, 2011).

Purpose and Hypothesis

The goal of the current study was to further explore the role of cerebellar Purkinje cells in respiratory compensatory responses by using a model mouse that exhibits a loss in function of Purkinje cells but not necessarily an absence of Purkinje cells. While DMD *mdx* musculature deficiencies do not appear until adulthood in mice (90 days postnatal), dystrophin expression, shown to be essential in Purkinje cell functioning, is inhibited from birth (Anderson, Head, & Morley, 2003, Jackson Laboratories, 2011; Snow, Fry, & Anderson, 2013; Vaillend et al., 2004). Preliminary research is currently being performed on the DMD *mdx* mouse investigating isolated hypoxic and hypercapnic conditions. Therefore the purpose of this study was to examine if cerebellar Purkinje cell dysfunction of the DMD *mdx* model mouse at 60 days of age contributes to delayed responses to challenges of a mixture of reduced oxygen and increased carbon dioxide content of the environment. Thus it was hypothesized that DMD *mdx* mutant mice would exhibit reduced compensatory responses (tidal volume and frequency) to isocapnic hypoxia compared to their wildtype, non-affected littermates.

CHAPTER 2: METHODOLOGY

Animals

This experiment used 14 male mice, seven DMD *mdx* mutants and seven wildtype (WT) littermate pairs, which are a recognized mouse model of DMD (C57BL/10ScSn-*Dmd*^{mdx}/J). Experimental subjects were bred and maintained in the Animal Care Facility located in the Department of Psychology at the University of Memphis. Mice were continuously maintained in a temperature controlled environment (21±1ºC) on a 12:12 light-dark cycle (lights on at 0700) and were given free access to food and water. Original DMD *mdx* (#001801) breeders were purchased from The Jackson Laboratory (Bar Harbor, Maine). All experiments were approved by the local Institutional Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. All mice were weaned between the ages of 21-27 days after birth and identified by numbered ear tags.

Procedure

The procedure followed the previously published methods used for the *Lurcher* mouse (Calton et al., 2014). The mice were exposed to the program randomly between 10am-6pm. Mice were weighed prior to placement in the chamber and their weights recorded for analysis. All experimental mice weighed between 26g +/- 3g. The equipment was disinfected and recalibrated between each trial. Additionally, the experimental room temperature (21°C +/-1°C) and humidity (25% +/-10%) were monitored daily to ensure stability throughout the experiment. Using Emka Technologies iOX2 software (WBP; Emka Technologies; Falls Church, VA, USA), one program was used to assess the subjects' respiratory responses at baseline (Normal room air; 21% O_2, 0%

CO_2 and 79% N_2), and under the condition where the assumed carbon dioxide pressure remains constant (isocapnia) while controlled reductions in oxygen were presented. Mice were randomly assigned to the isocapnic hypoxia program at 62 post-natal days (PND) following previous testing at 60 and 61 PND in isolated conditions of hypoxia or hypercapnia. Testing began with a 30-min habituation period, followed by a four-minute exposure to baseline (Room Air) period. Mice were exposed to continuously flowing room air at all times (0.8-1.0 L/m) during the habituation and baseline period to prevent fatal accumulation of CO_2.

Baseline. Upon completion of a the 30-min habituation period, dependent variables including tidal volume (TV; ml) and breath frequency (*f*; bpm), were continuously recorded while the mice were exposed to room air for a total of 4-min (21% O_2, 0% CO_2, 79% N).

Isocapnic hypoxia program. The entire program was 90 minutes long and consisted of one beginning baseline (30-min) measure as described above, followed by four sequential periods where CO_2 remained constant at 3%, (variable O_2, N_2 on balance). Each of the four O_2 challenges (19%, 17%, 15%, and 13%) consisted of a 2-min chamber fill period (the time required for the WBP to achieve the desired gas percentages) followed by a 4-min exposure measurement. To minimize discomfort of the animals, between each challenge the program returned to room air (again including a two-minute chamber refill period and a six-minute recovery period). At the termination of the final O_2 exposure (13%) and return to room air, the mouse was removed from the chamber (Appendix A).

Variables and Data Analysis

Animals. The weights of all animals (g) were recorded every day at testing for analysis. Because lung function is tightly correlated with body size, differences in weight would need to be treated as a covariate in subsequent analyses (Zosky, 2015).

The dependent variables (DVs) in all conditions were TV and *f*. TV was defined as the volume of inhaled air in one breath and *f* was defined as the number of breaths per minute. Both variables were averaged every 10 s across the entire program (both as received by the transducer and interpreted by the iOX2 software).

Baseline. The baseline measures of the mice collected prior to the experimental conditions were compared using analysis of variance (ANOVA). Measures of TV and *f* were separately analyzed. In both analyses Genotype (*MDX* and WT) served as the between- subjects factor while Interval (24, 10 s intervals) served as the within-subjects factor. Thus the omnibus analysis was a 2 (Genotype) x 24 (Interval) mixed design.

Isocapnic hypoxia. Two omnibus RMANOVAs were initially performed on TV and *f*. Genotype again served as the between subjects factor (*MDX* and WT). Within- subjects factors included four levels of Gas exposure (19%, 17%, 15%, and 13% O_2). Additionally, each gas exposure was subdivided into 2-min time blocks such that the blocks corresponded to seven conditions: chamber fill, gas exposure 1, gas exposure 2, chamber refill (return to room air), recovery 1, recovery 2, and recovery 3. The 2-min blocks were additionally divided into twelve, 10-s intervals in order to accurately track moment to moment changes in TV and *f*. Therefore the omnibus analysis was a 2 (Genotype) x 4 (Gas) x 7 (condition) x 12 (time block) mixed design.

Chapter 3: Results

Animal Weights

Independent samples *t*-test revealed no significant difference between the weights of the mutant mice and wildtype mice (*mdx* = 26.41g, *SEM* = .73; WT = 25.96, *SEM* = .52; t (12) = .508, p = .621). Thus, this variable was not included as a covariate in subsequent analyses.

Baseline

Breath Frequency. ANOVA indicated although breath frequency appeared lower in mutant mice (*MDX*, *M* = 289.86 bpm and WT, *M* = 331.51 bpm, *SEM* = 32.274 bpm) there was no significant difference between groups during baseline exposure to room air (Group, F (1, 12) = .810, p = .386; Figure 2A). Further, both groups remained equal over time blocks (Group x Time Block, F (23, 276) = .781, p = .563; Figure 2B).

Tidal Volume. A second ANOVA indicated that depth of breathing (TV) in both groups of mice (*MDX*, *M* = .180 mL and WT, *M* = .185 mL, *SEM* = .008 mL) did not differ during baseline either (Group, F (1, 12) = .169, p = .688; Figure 3A). Moreover, TV remained equivalent between groups through all of the time blocks (Group x Time Block, F (23, 276) = .453, p = .840; Figure 3B).

Isocapnic Hypoxia

Breath Frequency. ANOVA indicated that breath frequency in both genotypes was equivalent across all challenges (Group, F (1, 12) = 1.195, p = .296; data not shown). Further, both genotypes displayed the typical pattern of breath frequency increase with exposure to isocapnic hypoxia and decrease in breath frequency with return to room air, although these changes in patterns only approached significance (Condition, F (6, 72) = 2.200, p = .053; Figure 4).

Tidal Volume. A second ANOVA further revealed that both genotypes were equivalent on TV across all exposure conditions, (Group, F (1, 12) = .325, p = .579; data not shown). Further, both groups displayed a typical significant increase in TV upon exposure to isocapnic hypoxia along with a subsequent decrease in TV upon return to room air (Condition, F (6, 72) = 11.801, $p < .001$; Figure 5A) and this typical curvilinear response pattern was weakened with subsequently decreasing levels of O_2 (Gas x Condition, F (18, 216) = 1.714, p = .039; Figure 5B).

Chapter 4: Discussion

Baseline

At baseline the *mdx* mice and the WT controls had equivalent breath frequencies and tidal volumes when exposed to normal room air. These results suggest that the loss of dystrophin of *mdx* mice at PND 62 does not affect normal, rhythmic breathing (eupneic breathing). Further, these results support previous reports that diaphragm musculature failure in similarly aged *mdx* mice does not yet appear to inhibit these animals' ability to breathe under normal conditions (Mosqueira, Baby, Lahiri, & Khurana, 2013). It is worth noting that the current results do not support recent evidence that *mdx* mice have significantly lower breath frequencies than WT mice at eight weeks during baseline conditions, however there were numerous methodological variations between that study and the current study that could contribute to these differences (Burns, Edge, O'Malley, & O'Halloran, 2015). First, the current study evaluated seven sibling pairs (N = 7) while the previous study used 21 mice of unknown litter origin. Thus, it is possible the difference in N or possible litter effects could account for differences between these two studies. Second, each study defined baseline differently. The previous study combined two 10-min time periods, to create their baseline for comparison. The first time period consisted of 10, 1-min breath frequency averages prior to hypoxic exposure while the second time period followed exposure, after an unknown period of recovery. Combining both the baseline before and after exposure to the experimental program could have directly impacted their findings if the animals had not entirely recovered from the exposure period.

Isocapnic Hypoxia

Breath frequency between both genotypes was not different across challenges. Both groups increased their breath frequency when exposed to decreasing levels of O_2 and then decreased their breath frequency when placed back in normal room air. Tidal volume also remained similar between groups, both increased their depth of breathing when exposed to decreased O_2 and then decreased their TV upon return to room air. Previous research on DMD *mdx* has mainly focused on isolated hypoxia or hypercapnia challenges, but not a stepwise isocapnic hypoxia challenge. It is also important to note that although the results of the current experiment refute the exposure results of Burns et al. (2015), the program designs differed significantly. The current study used a stepwise isocapnic hypoxia protocol with intermittent returns to room air as opposed to Burns et al. (2015) who used a 20-min exposure to 10% hypoxia condition before administering an unknown length recovery of period. These differences may be of interest for further investigation in future research.

Relation to Previous Research

A reduction in cerebellar Purkinje cells is the most consistently reported neuropathology in autism spectrum disorder (ASD; Fatemi et al., 2012). While the *mdx* mutant mice do not exhibit cerebellar Purkinje cell loss, but rather dysfunction of these cells, it is important to note that dystrophin mediated dysfunction has also been identified in ASD (Kemper & Bauman, 2002). Cerebellar Purkinje cells and their effect on various behavioral functional domains and learning tasks have been explored in previous research using mouse models with a range of cerebellar Purkinje cell loss from 0%-100% (Hampson & Blatt, 2015). A series of experiments found that developmental loss of Purkinje cells resulted in significant deficits in executive

functions through the results of lever pressing, open field activity, and nose poking in exploration tasks (Martin, Goldowitz, & Mittleman, 2010). Another study analyzing a mouse model of tuberous sclerosis complex, a genetic disorder with a high rate of autism spectrum disorder, supports findings that Purkinje cell dysfunction results in abnormal social behaviors and cognitive behaviors related to motor learning (Tsai et al., 2012). Additionally, Purkinje cell degeneration and subsequent dysfunction in rats was observed to inhibit modulation of eupneic and hypercapnic ventilation (Xu et al., 2004). Both Purkinje cells loss and Purkinje cell dysfunction appear to result in similar behavioral and executive function changes, in addition to abnormally regulated ventilatory responses that can result from the impairment of cognitive networks located in the cerebellum (Tsai et al., 2012).

Previous research has reported a functional deficit in Purkinje cells associated with the loss of postsynaptic dystrophin in *mdx* mice (Anderson et al., 2003). While *Lurcher, (Lc/+*), mutant mice exhibit global loss of cerebellar Purkinje cells that begins almost immediately after birth, with nearly 100% Purkinje cell loss in the cerebellar cortex by postnatal day 28, studies on Purkinje cell dysfunction have shown similar changes in behavior and ventilation responses (Caddy & Biscoe, 1979; Cendelin & Vozeh, 2013; Kemper & Bauman, 2002; Tsai et al., 2012; Xu et al., 2004; Zuo et al., 1997). Unlike the *mdx* mice in this study, response patterns indicated that *Lc/+* mice were impaired in responding to multiple aspects of the CO_2 challenge (Calton et al., 2014). *Lc/+* mice were slower to initiate compensatory respiratory responses to hypoxia and hypercapnia, but were able to achieve the ventilatory levels of the WT mice as the challenges increased (Calton et al., 2014). Similarly, research has found significant differences between *mdx* and WT mice ventilatory responses to hypoxic stress, and it was observed that the *mdx* mice could match the results found in the WT mice in regards to ventilation capacity at eight weeks of

age (Burns et al., 2015). While the current results did not find differences during normal breathing or during a mixed challenge, the combination of the two gasses occurring in the challenge of isocapnic hypoxia could account for this, at least in part.

The dual challenge of isocapnic hypoxia could present simultaneously occurring reactions which could make identifying significant differences between the animals tested difficult. Previous studies have typically analyzed the single challenge of an increase in carbon dioxide or the single challenge of a decrease in atmospheric oxygen content. Many studies analyzing a mixture of hypoxia and hypercapnia studied the effects of high altitude during hypercapnia and the physiological responses involved in a natural occurring situation where the human body is frequently exposed to low levels of oxygen (Lambertsen, 1971). The combination of these two types of situational responses may mask differences in response patterns.

In a random population sample of cognitively normal humans, hypoxic and especially hypercapnic challenges resulted in a significant increase in minute ventilation compared to normal room air (Somers, 1989). Contrastingly, the current study did not reveal significant differences in minute ventilation between the alternating concentrations of gases in the combined challenge of isocapnic hypoxia. While both results of the hypercapnia challenge and the hypoxia challenge were significant compared to controls, the isocapnic hypoxia challenge in the previous human study had both a simultaneous increase on sympathetic excitation and a decrease in minute ventilation between the two challenges compared to a single challenge (Somers, 1989). The previous study on humans establishes a baseline for what should occur within a normal population given the challenge of isocapnic hypoxia. The combination of the two gas challenges in the current study could have a similar effect of both decreasing and increasing ventilation responses simultaneously.

Implications for Future Research

Future research on cerebellar Purkinje cell deficiency and the effect on normal ventilation in the mouse model remains warranted despite the current results not revealing differences in response to a mixed stressor. It is possible that individuals chronically exposed to hypoxia or hypercapnia would reveal respiratory responses to an isocapnic hypoxia challenge much differently than those acutely exposed. However, to isolate differences, future research should focus on a single challenge of hypoxia or hypercapnia in potentially vulnerable populations. Further research to explore dystrophin deficiency and loss in Purkinje cell function would assist researchers in exploring additional management of the disease in patients as well as developing effective treatments for respiratory therapy. Fatemi (2012) suggested Purkinje cell loss, and subsequent dysfunction, may have further effects on cerebellar malformation. Future research could explore whether loss and subsequent dysfunction or dysfunction alone of cerebellar Purkinje cells causes any possible cerebellar malformation and subsequent increases in dysfunction of the cerebellum during development.

Conclusion

The current study was designed to further explore the role of cerebellar Purkinje cells in respiratory compensatory responses by using a mouse model that exhibits a loss in function of Purkinje cells but not necessarily an absence of Purkinje cells. It was hypothesized that DMD *mdx* mutant mice would exhibit reduced compensatory responses (tidal volume and breath frequency) to isocapnic hypoxia compared to their wildtype, non-affected littermates. No significant differences were found between the DMD *mdx* and wildtype mice, but it is likely the mixture of gasses mitigated the ability to detect differences between these two groups. Future

research should focus on single-challenge tests of hypercapnia and hypoxia to eliminate this confound.

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Appendix A

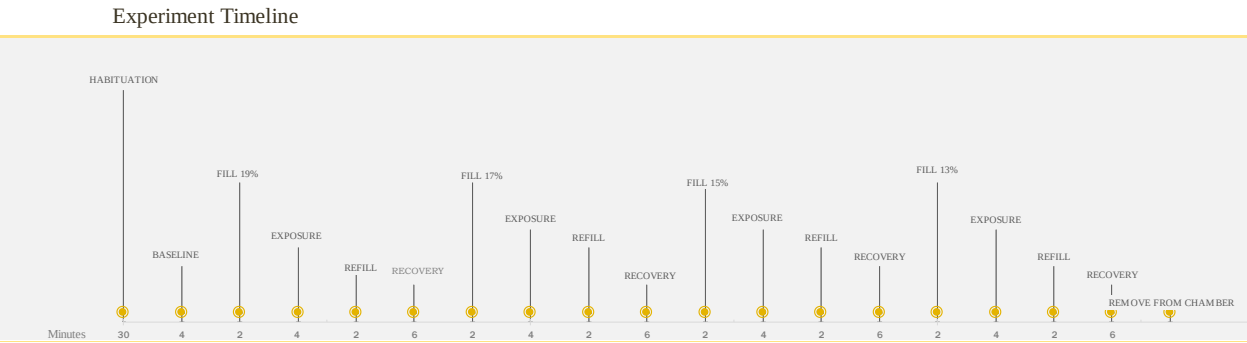

Figure 1. Experiment timeline. Testing began with a 30-minute habituation period, followed by a four-minute exposure to baseline (Room Air) period. Gas fill was divided into two-minute time blocks, with each sequence of fill and exposure having a two-minute fill period followed by a four-minute exposure period. Each sequence was followed by a two-minute refill to normal room air, followed by a four-minute recovery period before the start of the next level of gas exposure. Mice were removed from the chamber at the end of testing.

Appendix A

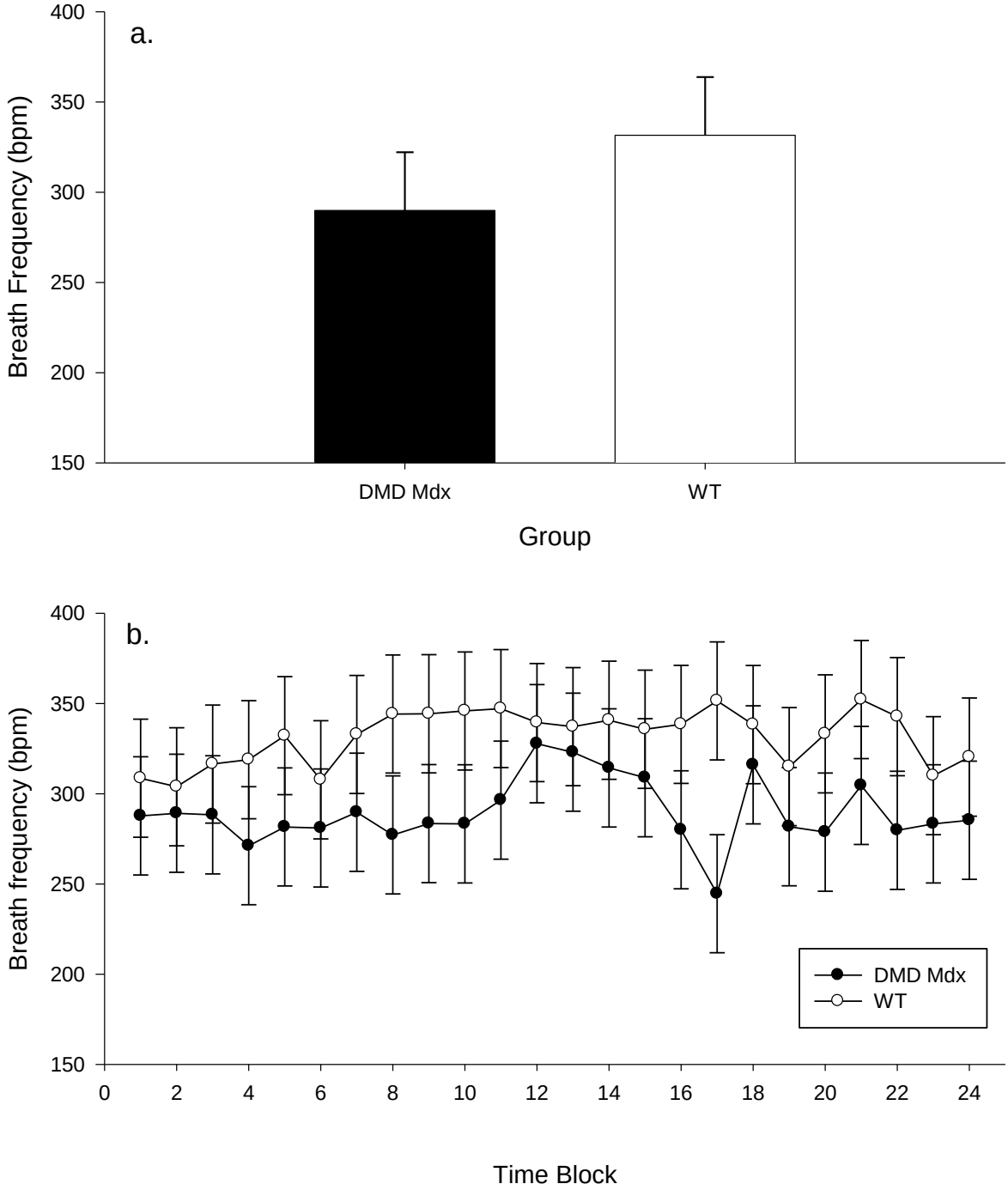

Figure 2. Breath frequency at baseline from two sibling paired groups of *MDX* mice day 60, 61, and 62 PND when exposed to normal room air for 4 minutes. (A) There was no significant difference between groups during baseline exposure to room air. (B) Also, both groups remained consistently equal over time blocks.

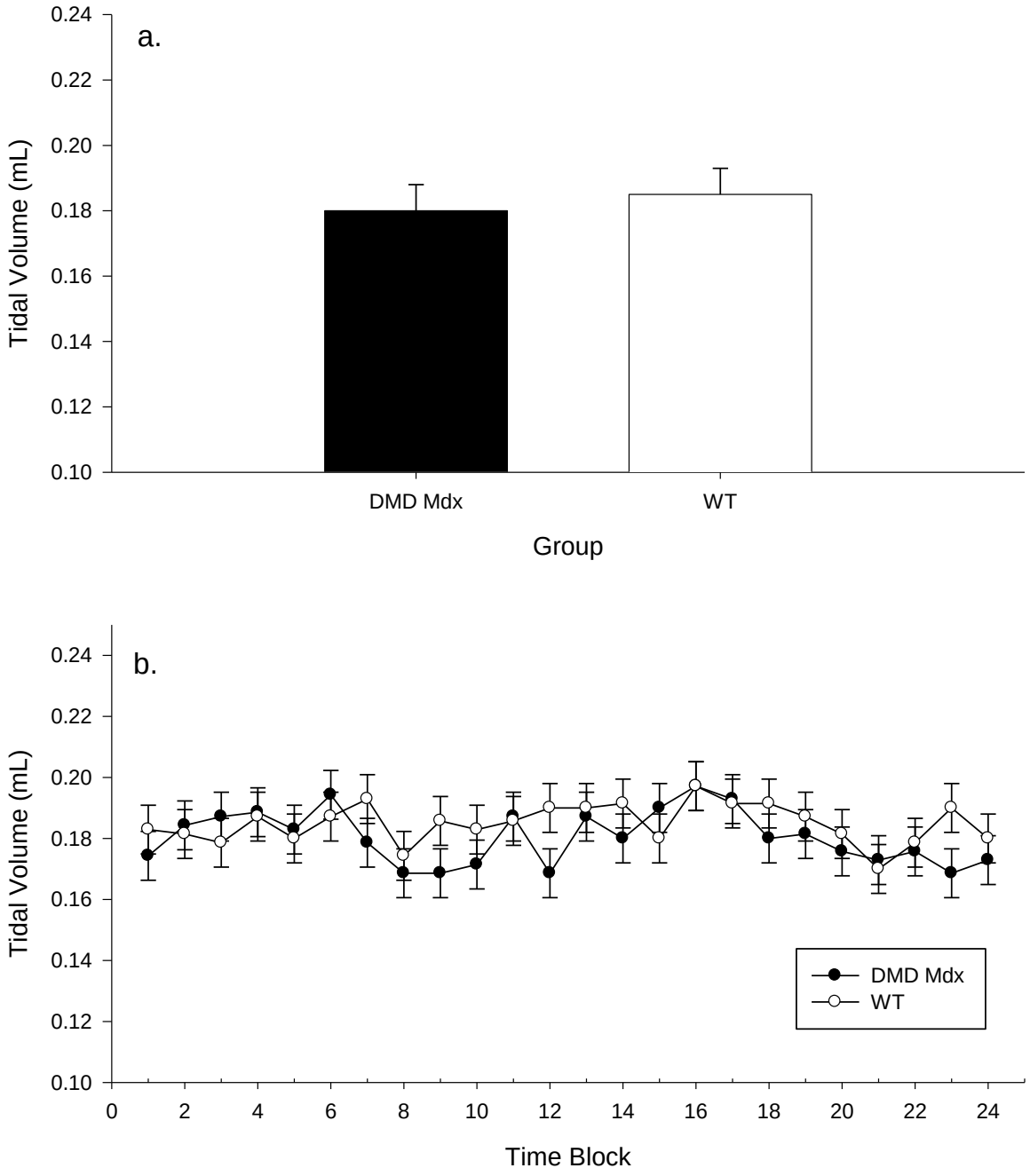

Figure 3. Tidal volume at baseline from two sibling paired groups of *MDX* mice day 60, 61, and 62 PND when exposed to normal room air for 4 minutes. (A) Tidal volume did not significantly differ between groups during baseline exposure to room air. (B) TV remained equivalent between groups through all time blocks during baseline exposure to room air.

Appendix A

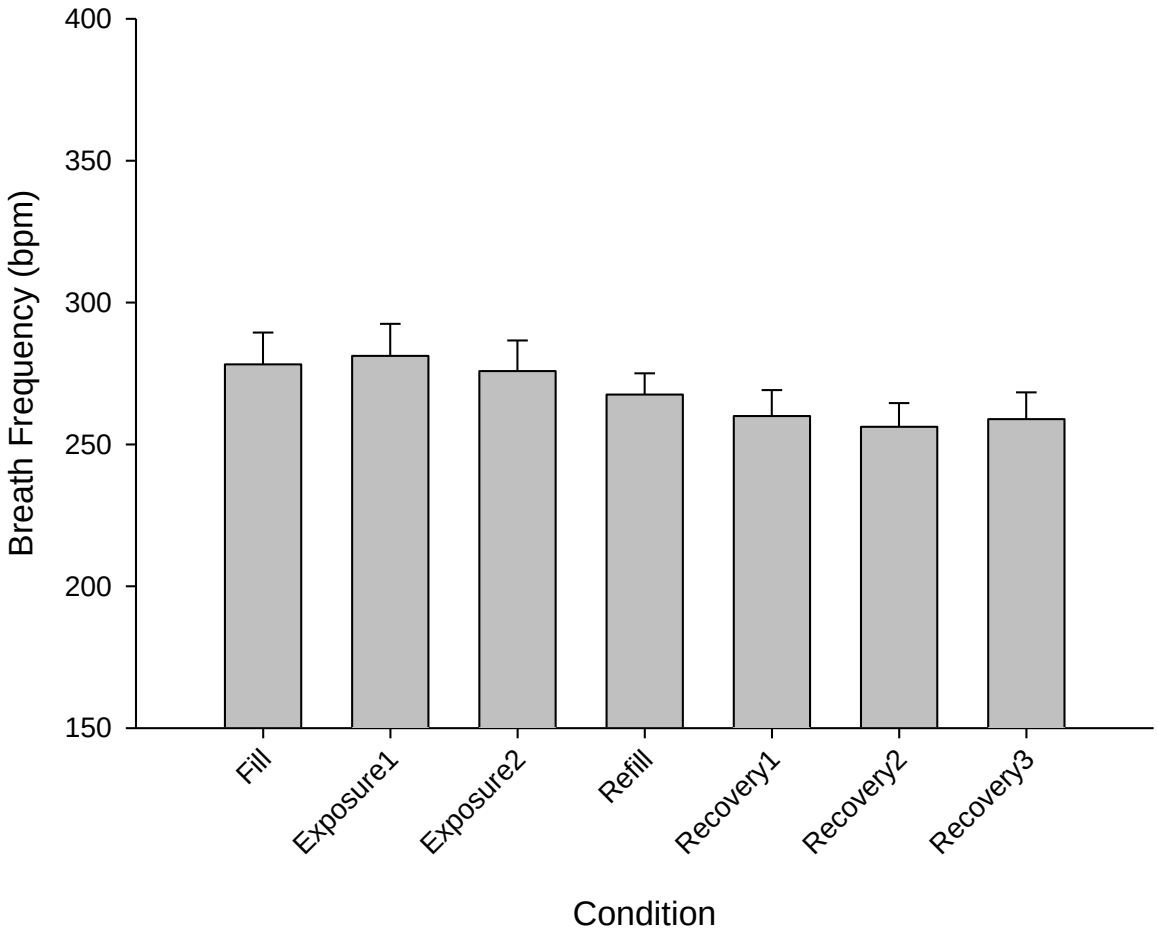

Figure 4. Breath frequency during isocapnic hypoxia challenges in both genotypes of *MDX* mice day 60, 61, and 62 PND. Both genotypes displayed the typical pattern of decreasing breath frequency with decreasing levels of O_2 combined with 3% CO_2, but changes only approached significance.

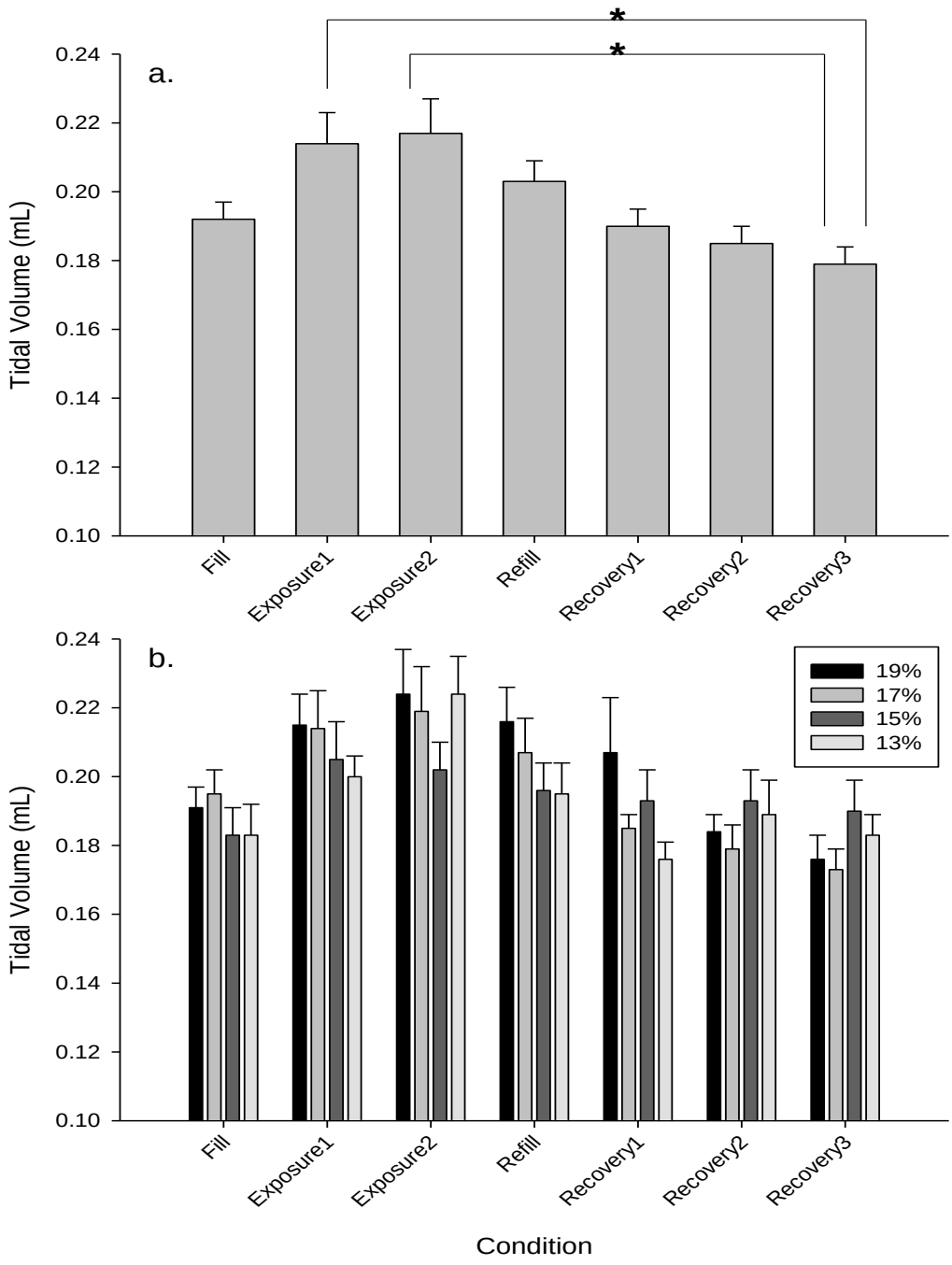

Figure 5. Tidal volume during isocapnic hypoxia challenges in both genotypes of *MDX* mice day 60, 61, and 62 PND. (A) Both groups displayed a typical significant increase in TV during the isocapnic challenge along with a subsequent decrease in TV when returned to room air. (B) The response pattern was intensified with decreasing levels of O_2.

THE UNIVERSITY OF MEMPHIS

IACUC PROTOCOL ACTION FORM

To:	Guy Mittleman
From	Institutional Animal Care and Use Committee
Subject	Animal Research Protocol
Date	3-18-13

The institutional Animal Care and Use Committee (IACUC) has taken the following action concerning your Animal Research Protocol No.

0721 (Mouse model of SIDS …)

☒ Your proposal is approved for the following period:

From: March 18, 2013 To: March 17, 2016

☐ Your protocol is not approved for the following reasons (see attached memo).

☐ Your protocol is renewed without changes for the following period:

From: To:

☐ Your protocol is renewed with the changes described in your IACUC Animal Research Protocol Revision Memorandum dated for the following period:

From: To:

☐ Your protocol is not renewed and the animals have been properly disposed of as described in your IACUC Animal Research Protocol Revision Memorandum dated:

Prof. Guy Mittleman, Chair of the IACUC

Dr. Karyl Buddington, University Veterinarian
And Director of the Animal Care Facilities