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Compensatory Respiratory Effects in Three Mouse Models of Developmental Cerebellar Neuropathology during Exposure to and Recovery from Hypoxic and Hypercapnic Challenges

Michele Ann Calton

COMPENSATORY RESPIRATORY EFFECTS IN THREE MOUSE MODELS OF DEVELOPMENTAL CEREBELLAR NEUROPATHOLOGY DURING EXPOSURE TO AND RECOVERY FROM HYPOXIC AND HYPERCAPNIC CHALLENGES

by

Michele A. Calton

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Dedications

I dedicate this dissertation to my husband, John Calton, for his unwavering support and continued encouragement in everything I do. He has been my rock throughout this process, keeping me grounded even during the times I felt completely overwhelmed. The work presented in this dissertation is the direct product of both hard work and his support—I could not have made it this far without everything he has done and foregone. I love you John.

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Abstract

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Sudden unexplained death occurs in multiple populations. Significant evidence suggests fatality was due to cardiorespiratory failure—likely in response to an exogenous stressor which created an immediate hypercapnic (increased carbon dioxide) or hypoxic (decreased oxygen) environment. Research into the mechanism(s) of failure has focused on the brainstem (the classic cardiorespiratory control center) to no avail. Recently, research has probed outside of the brainstem to identify structures that may play a specific modulatory role during stressful conditions such as hypoxia and hypercapnia, for example the cerebellum, which houses a pair of chemosensitive nuclei, the fastigial nuclei (FN). As the Purkinje cells of the cerebellar vermis primarily innervate the FN, and global cerebellar Purkinje cell loss inhibits the ability to respond to and recover from mild hypoxic and hypercapnic stressors, the purpose of this study was to examine the respiratory responses of multiple animal models of developmental cerebellar Purkinje cell loss or dysfunction to hypoxic and hypercapnic stressors. It was hypothesized that cerebellar Purkinje cell loss or dysfunction, regardless of extent, would result in a reduced ability to respond to and recover from hypoxic and hypercapnic stressors. Twelve male sibling mutant and wildtype mouse pairs from three strains (*Fmr1*, *Lurcher*, and *mdx*) on three different genetic backgrounds (FVB, B6, and B10 respectively) were exposed to multiple environmental stressors with periods of recovery in normal room air in a whole body plethysmography system. Respiratory minute ventilation (MV) patterns

were examined for changes in depth of breathing, breath frequency, and pausing between breaths. Results of multiple mixed analyses of covariance indicated all animals responded accordingly to the hypoxic and hypercapnic stressors by increasing their MV and subsequently decreasing MV when allowed to recover in normal conditions. However, regardless of strain, all mutant animals with cerebellar Purkinje cell loss or dysfunction were slower to respond to stressors and revealed patterns of disordered breathing during recovery. As cerebellar Purkinje cell loss or dysfunction and disordered breathing are common in multiple populations at risk for sudden unexplained death, the precise role of this neuropathology in the fatal event of sudden death victims should be further investigated.

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Key to Abbreviations and Symbols

5-HT	Serotonin
AEDs	Anti-Epileptic Drugs
ANOVA	Analysis of Variance
ASDs	Autism Spectrum Disorders
CNS	Central Nervous System
CO_2	Carbon Dioxide
DCN	Deep Cerebellar Nuclei
DMD	Duchenne's Muscular Dystrophy
DV	Dependent Variable
EEP	End Expiratory Pause
f	Breath Frequency
Fmr1	Fragile Mental Retardation Mouse Model
FMRP	Fragile Mental Retardation Protein
FN	Fastigial Nuclei
FXS	Fragile X Syndrome
GABA	γ-aminobutyric acid
GRID2	Glutamate Receptor, Ionotropic, Delta 2 Gene
IV	Independent Variable
mdx	Duchenne's Muscular Dystrophy Mouse Model
MV	Minute Ventilation
N_2	Nitrogen
O_2	Oxygen
PCR	Polymerase Chain Reaction
PND	Post Natal Day
RMANCOVA	Repeated Measures Analysis of Covariance
SIDS	Sudden Infant Death Syndrome
SUDC	Sudden Unexplained Death in Childhood
SUDEP	Sudden Unexplained Death in Epilepsy
SUDS	Sudden Unexplained Death Syndrome
TV	Tidal Volume
WBP	Whole Body Plethysmography

Chapter 1: Introduction

Sudden Unexplained Death

Sudden unexplained death occurs in multiple populations including infants (sudden infant death syndrome; SIDS), persons with epilepsy (sudden unexplained death in epilepsy; SUDEP), children (sudden unexpected death in childhood; SUDC), and adults (sudden unexpected death syndrome; SUDS) among others, and is a pervasive problem that demands solving. In each of these cases, the victims appeared otherwise healthy prior to the fatal event, are typically males, and are found in bed in the prone position suggesting the distinct possibility that the fatal event has occurred during sleep (Krous et al., 2004; Moon, Horne, & Hauck, 2007; NICHD, 2001). Diagnosis for each of these disorders is exclusionary—no known cause of death is found even following a full investigation and autopsy. Although the precise cause of death is not known across these populations, there are often factors surrounding the deaths that have led many investigators to conclude that cardiorespiratory failure occurred, culminating in the fatal event. In addition to inherited vulnerabilities there are often interesting and unifying neuropathological findings during histological examination of the brains of these victims that suggest that there may be some commonality in the neural mechanisms contributing to the vulnerability to these disorders.

Sudden infant death syndrome. SIDS is defined as the sudden, unexpected death of an infant less than 1 year of age (Krous et al., 2004). SIDS risk vulnerability begins shortly after birth, with few deaths occurring before one month of age, and peaks between two and four months of age (Task Force on Sudden Infant Death Syndrome, 2005). The principal explanation for the cause of SIDS, the triple-risk hypothesis, is multifactorial

and states that in order to be identified as a SIDS victim three factors must converge: an infant must have been at a critical period of development, have an unknown brain abnormality, and been exposed to an exogenous stressor (Filiano & Kinney, 1994; NICHD, 2001). Of the three factors involved in the triple-risk SIDS hypothesis, exogenous stressors are unique in that researchers have been able to specifically identify and educate the public about many of these stressors and subsequently reduce overall SIDS deaths (Task Force on Sudden Infant Death Syndrome, 2005; Trachtenberg, Haas, Kinney, Stanley, & Krous, 2012). Because the majority of SIDS deaths are thought to occur during sleep, investigation of exogenous stressors is typically focused on stressful sleeping conditions such as prone sleeping, or sleeping in soft bedding. These stressors are known to either increase the infant's immediate environmental levels of carbon dioxide (CO_2), or decrease available oxygen (O_2), or both. Although the identification of these exogenous stressors has, over two decades, reduced SIDS deaths, this condition remains the leading cause of unexplained death in infants, which affirms the importance of investigating and understanding the factors involved in SIDS vulnerability (Hauck & Tanabe, 2008; Moon et al., 2007; NICHD, 2001; Trachtenberg et al., 2012).

Considerable evidence has suggested that SIDS occurs due to cardiorespiratory failure. This is a reasonable conclusion as the stressors an infant may be exposed to cause changes in the infant's environmental levels of CO_2 and O_2 that challenge homeostatic cardiac and respiratory mechanisms (e.g., sleeping in soft bedding, in the prone position, etc…). Accordingly, research has focused on the cardiac and pulmonary musculature and related brain systems involved in maintaining and regulating cardiorespiratory function. Specific attention has been paid to pontine and medullary regions of the hindbrain as they

have been established as control centers of normal, rhythmic respiratory and cardiac output (Bianchi, Denavit-Saubie, & Champagnat, 1995). However, when these systems are challenged (e.g.,, an increase in environmental CO_2), additional central nervous system (CNS) structures may be recruited to initiate and maintain appropriate compensatory responses including increased respiration, increased heart rate, repositioning, or even arousal. Thus, only recently has SIDS research extended into investigation of neural systems outside of the brainstem into brain areas including the cerebellum, which may modulate cardiorespiratory behavior under challenging or stressful conditions (Harper, 2000).

The influence of the cerebellum in SIDS remains controversial with mixed support for the hypothesis that this brain area plays a causal role in fatal events. For example, while Cruz-Sanchez et al. (1997) found evidence of delayed cerebellar cortex maturation in SIDS victims compared to controls, two additional reports found no difference in cerebellar pathology between SIDS victims and controls (Kiessling et al., 2013; Oehmichen, Wullen, Zilles, & Saternus, 1989). It is important to note in the two non-supporting studies that the hypoxic conditions (e.g.,, pneumonia, asphyxia, suffocation, carbon monoxide poisoning) that preceded many of the control deaths may have contributed to the inability to find these differences as it has been shown repeatedly that the infant cerebellum is especially vulnerable to hypoxic insult resulting in neuronal death (Campanille et al., 2015, Huang & Castillo, 2008; Sarna & Hawkes, 2003). Additionally, because the infant cerebellum is especially vulnerable to hypoxic insult, these studies cannot differentiate the possibility that pre-existing cerebellar neuropathology led to the fatal event from the competing hypothesis that episodes of

recurrent non-fatal hypoxia ultimately led to the fatal event, but also resulted in hypoxic cerebellar damage. Because of likely variability in the many factors associated with autopsy procedures it is also possible that the observed cerebellar hypoxic damage is tied to variables related to the time interval between the fatal event and discovery, or possibly the duration of the fatal event.

Sudden unexplained death in epilepsy. SUDEP is defined as unexplained death in persons with epilepsy who (aside from seizures) appeared otherwise healthy before the fatal event (Nashef, 1997). Deaths that can be attributed to secondary events that unfold relative to ictal or postictal periods (e.g., hitting head, drowning, etc…) are not included in a SUDEP diagnosis. Similar to SIDS, research has reported elevated risk for SUDEP in males [with epilepsy], during sleep, with victims often found deceased in the early morning hours in bed, and in the prone position (Kloster & Engelskjøn, 1999; Tomson, Nashef, Hindocha, & Makoff, 2007). Additional risk factors for SUDEP include a history of multiple monthly seizures, especially grand-mal seizures, middle age (20 – 40 years), polytherapy with multiple antiepileptic drugs (AEDs), poor compliance with AEDs, alcohol abuse, and intellectual disabilities including autism spectrum disorders (ASD) which frequently present comorbidly with epilepsy (Hughes, 2009; Kiani et al., 2014; Kloster & Engelskjøn, 1999). Hypotheses for the causes of SUDEP again emphasize the likelihood of cardiorespiratory failure. Thus, investigation into the causes of SUDEP has also focused extensively on the brainstem. It should be noted, however, that neuropathological findings from SUDEP victims have suggested the necessity of examining areas outside the brainstem in an effort to find causal influences on the susceptibility for SUDEP.

Cerebellar pathology including atrophy and Purkinje cell loss is common in both patients with chronic epilepsy and victims of SUDEP (Botez, Attig, & Vezina, 1988; Dam, 1987; Thom et al., 2015). Neuropathology of the cerebellum has often been attributed to the reoccurrence of grand-mal seizures resulting in hypoxic brain injury, and the use of antiepileptic drugs such as phenytoin has been shown to induce cerebellar cell death (Botez et al., 1988; Brostoff, Birns, & McCrea, 2008; Dam, 1987; Eldridge, Stern, Anainen, Koerber, & Wilder, 1983; Mclain, Martin, & Allen, 1980). However, even without AEDs, clinical research indicates that cerebellar pathology is much more common in patients with epilepsy and is related to seizure frequency (Bohnen, O'Brien, Mullan, & So, 1998). Preclinical research also confirms a significant relationship between seizures and neuronal loss in the cerebellum (Dam et al., 1984; Lomoio et al., 2011). More specifically, increased severity and frequency of seizures results in decreased cerebellar Purkinje cell density (Lomoio, Necchi, Mares, & Scherini, 2011). Thus, cerebellar neuropathology can occur as a function of AED treatment or from the repeated hypoxic episodes that accompany seizures.

Sudden unexplained death in childhood. SUDC is the unexplained death of a child greater than 1 year of age extending up until 18 years of age (Hefti et al., 2016; Krous, Chadwick, Crandall, & Nadeau-Manning, 2005). Similar to SIDS, these deaths primarily occur in males during sleep and the victims are often found in the prone position, again raising the possibility these deaths could have arisen from acute cardiorespiratory failure. However, the phenomenon of SUDC has not been as extensively reported as SIDS and thus knowledge is limited. Recent research on 151 referred children victims in San Diego who perished from known causes, unknown causes, seizure-related causes, and SUDC

has provided little insight into identifying unifying features of SUDC, but did reveal the necessity of instituting a nationwide autopsy protocol to further the knowledge on SUDC (Hefti et al., 2016).

Sudden Unexpected Death Syndrome. Unlike SIDS, SUDEP, and the more recently described SUDC, SUDS has yet to be universally defined. The suggested windows of time that characterize this sudden death syndrome have been variably described as above the age of one year (Morentin, Suárez-Mier, & Aguilera, 2003), at least the age of two years (Tungsanga & Sriboonlue, 1993), between the ages of 20-39 (Gervacio-Domingo, Punzalan, Amarillo, & Dans, 2007), and young adult below the age of 40 (Elfawal, 2000). Further there is inconsistent use of terminology to describe this syndrome from the addition of the term "nocturnal" to describe *sudden unexpected nocturnal death syndrome*, to interchanging of the terms unexpected and unexplained, the omission of both terms (unexpected and unexplained) altogether referring to it simply as sudden death syndrome, or even the reference to "dead in bed syndrome" which is most frequently used to describe sudden unexplained death in diabetes patients. Similar to SIDS, SUDEP, and SUDC, most victims of SUDS are males found in bed following periods of sleep, and complete autopsies and scene investigations yield no causal factors of the death (Elfawal, 2000; Furst, 1982; Gervacio-Domingo et al., 2007; Tungsanga & Sriboonlue, 1993). As the most common cause of sudden deaths in young adults is the result of underlying previously unknown cardiopathology (Corrado, Basso, & Thiene, 2001: Maron et al., 1996), much of the research has been focused on genetic cardiovascular pathophysiological risk factors for cardiac malfunction with respiratory risk factors rarely investigated. As SIDS, SUDEP, SUDC, and SUDS each appear to have overlapping

features with delineation arbitrarily made by age, it may be beneficial to model future SUDS research after more extensively studied sudden death syndromes.

Eupneic and Stressed Breathing

Relaxed breathing (eupneic breathing) occurs during normally oxygenated environmental conditions (21% oxygen, O_2; 79% nitrogen, N_2). Respiration is defined by a number of accepted breathing characteristics that include minute ventilation (MV), which is the amount of air breathed per minute. Minute ventilation is the mathematical product of depth of breathing (tidal volume, TV) and frequency of breathing (*f*). The frequency of breathing includes many components of respiration that all contribute to the overall number of breath oscillations that can occur in one minute. One of these components is TV; the larger the TV, the longer it may take to inspire, and thus the lower the breath frequency could be (although the individual could simultaneously decrease their inspiratory time mitigating the effect of this increase in TV on *f*). Another interesting component of breath frequency is the end expiratory pause (EEP). The EEP is the time period that occurs following exhalation and before inhalation. EEP is an important breathing component in examining apnea, which is an extended EEP period that varies slightly in definition depending on species and is an indicator of disordered breathing and a reduced ability to compensate for rising blood CO_2 levels (Feldman, Mitchell, & Nattie, 2003; Nattie, 1999). During normal eupneic breathing EEP periods consist of the minimal amount of time needed for the body to initiate and complete a reversal in breathing function from exhalation to inhalation. It is widely accepted that eupneic breathing is initiated and maintained by the pons and medullary structures of the hindbrain (Feldman et al., 2003).

Stressed breathing occurs during challenging conditions of increased carbon dioxide (hypercapnia) or decreased levels of oxygen (hypoxia). Under these challenges compensatory respiratory patterns occur which increase overall MV via increases in TV, *f*, or both as EEP periods decrease. Such compensatory changes are crucial to reducing rising blood CO_2 levels and increasing blood O_2 levels. Typically as environmental CO_2 increases, and thus blood levels of CO_2 increase, the phenotypic compensatory response is to increase breath frequency in an effort to expel the excess CO_2. Shorter EEP periods facilitate this effort by reducing the amount of time of CO_2 accumulation in the body before reintroducing O_2 with inhalation. Hypoxia elicits a similar MV compensatory response, but the increases are typically the result of amplified TVs in an effort to absorb more available O_2. Failure to respond appropriately to hypercapnic or hypoxic conditions by increasing MV while maintaining or decreasing EEPs will accordingly result in an increased body burden of CO_2 with less available O_2 for normal cellular functioning, with potentially fatal consequences (Moore, Zwillich, Battaglia, Cotton, & Weil, 1976).

Because it is well established that brainstem structures are responsible for eupneic breathing they have also served as the primary focus of investigation for failures of stressed breathing. Given the apparently consistent involvement of the cerebellum in multiple conditions involving sudden death it seems reasonable to expand the scope of investigation outside of the traditionally implicated brainstem structures, into other brain regions that have potential involvement in the regulation of stressed breathing. Specifically, it is possible that the cerebellum, a structure massively innervated by the pons and medullary brainstem regions, may play a modulatory role in cardiorespiratory compensatory responses to environmental challenges.

The Cerebellum

The cerebellum, also located in the hindbrain, has been well established as a structure importantly involved in motor control (Fine, Ionita, & Lohr, 2002; Manto et al., 2012). It is generally regarded that the cerebellum is responsible for the smooth, fluid-like coordination of voluntary movements as damage to this structure results in uncoordinated and ataxic behaviors proportional to the level of damage (Richter et al., 2005). For many years, research on the cerebellum has remained confined within the realm of movement disorders. Recent research, however, has implicated this structure in multiple functions outside of motor control including cognitive abilities and cardiorespiratory modulation (Calton, Dickson, Harper, Goldowitz, & Mittleman, 2014; Dickson et al., 2013; Dickson et al., 2010; Fatemi et al., 2012).

The cerebellum appears to play little to no role in cardiorespiratory functioning during eupneic, relaxed breathing. Following complete cerebellectomy, eupneic patterns of respiration and blood pressure are unchanged (Janssen, Lutherer, & Barnes, 1981; Xu & Frazier, 1997; Xu, Owen, & Frazier, 1995; Xu, Taylor, Lee, & Frazier, 1993). The cerebellum, however, does appear to play an important role in respiratory responding under stressed conditions. Following complete cerebellectomy, respiration under hypoxic conditions is impaired as indicated by decreased minute ventilation, TV and f (Xu et al., 1995).

One potential explanation for the involvement of the cerebellum in cardiac and respiratory responses in stressed conditions may involve one of the deep cerebellar nuclei (DCN), the fastigial nuclei (FN). The FN has been identified as a chemosensitive area of the brain, especially responsive to changes in blood levels of CO_2 (Lutherer, Williams, &

Everse, 1989; Xu & Frazier, 2002). The FN appears to play a modulatory role in both cardiac and respiratory responses. In anesthetized cats, stimulation of the FN, but not the other cerebellar nuclei resulted in increases in blood pressure (Achari & Downman, 1970). Further, low frequency electrical stimulation of the FN elicited an excitatory respiratory response in cats, while high frequency stimulation resulted in an inhibition of respiratory response with an increase in apneic episodes (Williams, Everse, & Lutherer, 1989).

Involvement of the FN in cardiac and respiratory modulation appears specific to conditions of stress but not during eupneic breathing. Cell body lesions of this structure exerted no effect on normal respiratory patterns or arterial blood pressure (Takahashi et al., 1995). Under hypercapnic conditions, bilateral lesions of the FN resulted in an attenuated respiratory response to increasing levels of CO_2 (Xu & Frazier, 2000; Xu et al., 1994) and when challenged with hypotension inducing drugs, heart rate and blood pressure recovery were additionally and significantly impaired (Chen & Lutherer, 1985; Chen, Williams, & Lutherer, 1994; Lutherer, Lutherer, Dormer, Janssen, & Barnes, 1983; Williams, Robinson, & Lutherer, 1986).

The FN is primarily innervated by the Purkinje cells of the cerebellar vermis (Kandel, 2000; Lu, Cao, Tokita, Heck, & Boughter, 2013; Zhang, Wang, & Zhu, 2016). Purkinje cells comprise the sole output of the cerebellum and global loss of these cells inhibit the ability of mice to recover appropriately from mild carbon dioxide exposure (Calton et al., 2014; Calton, Howard, Harper, Goldowitz, & Mittleman, 2016). Further, decreased cerebellar Purkinje cell number and density, especially in the vermis, is the most frequent finding in persons diagnosed with ASD—a population with a high

incidence of disordered breathing and a rate of mortality twice that of the general population (Fatemi et al., 2012; Mouridsen, Brønnum-Hansen, Rich, & Isager, 2008; Palmen, Engeland, Hof, & Schmitz, 2004; Pickett, Paculdo, Shavelle, & Strauss, 2006; Shavelle, Strauss, & Pickett, 2001).

Additionally, the cerebellum is richly innervated by serotonin (5-hydroxytryptophan; 5-HT) projections and although the precise role is evidently complex, it has been suggested that 5-HT has a direct influence on deep cerebellar nuclei firing (Saitow, Hirono, & Suzuki, 2013) which would include the FN. Although the relationship between 5-HT and its effects in the cerebellum is incredibly complex both temporally and spatially, excessive cerebellar 5-HT has been shown to impair timing signals of the deep cerebellar nuclei (Saitow, Murano, & Suzuki, 2009) and blood hyperserotonemia has been reported in ASD patients although the central effects remain largely unknown (Whitaker-Azmitia, 2005). Further, in the *Lurcher* mouse, an animal model of cerebellar Purkinje cell loss (the most common finding in ASD), increased serotonin projections to the DCN have been observed which may suggest a rewiring of the 5-HT network in these cerebella (LeMarec, Hebert, Amdiss, Botez, & Reader, 1998; Strazielle, Lalonde, Riopel, Botez, & Reader, 1996). As serotonin is widely accepted as integral in respiratory modulation, and neuropathophysiogical changes in the number or distribution of 5-HT are suspected as influential in sudden unexplained death disordered breathing patterns (Feldman et al., 2003; Harper & Kinney, 2010; Richerson & Buchanan, 2011; Richter, Mazke, Wilken, & Ponimaskin, 2003), it may be necessary to investigate this complex relationship between cerebellar neuropathology including Purkinje cell loss or dysfunction, central serotonin, ASD symptomology, and breathing.

Although, to date, there have been no systematic investigations into unexplained deaths in the ASD population, mouse models with similar cerebellar neuropathology may help shed light on the possibility of vulnerability to unexplained death in this group.

Animal Models of Developmental Cerebellar Neuropathology

***Fmr1*.** Fragile mental retardation 1 (*Fmr1*) is a gene that encodes for fragile mental retardation protein (FMRP; Hagerman & Hagerman, 2002). Increased expression of the trinucleotide (CGG) segment of the *Fmr1* gene results in silencing of the gene, which further results in a stop to FMRP production—key deficits in Fragile X syndrome (FXS). FXS is the most commonly inherited genetic cause of intellectual disability with up to 33% of those afflicted meeting criteria for diagnosis of ASD (Rogers, Wehner, & Hagerman, 2001) and up to 25% being diagnosed with epilepsy (Berry-Kravis, 2002; Wisniewski, Segan, Miezejeski, Sersen, & Rudelli, 1991).The *Fmr1* mouse also exhibits a silencing of the *Fmr1* gene halting FMRP production and has been a widely used model for investigating the physiological and behavioral effects of FXS and autism (Bakker et al., 1994; Pietropaolo, Guilleminot, Martin, D'Amato, & Crusio, 2011).

Although there is little information about the likelihood of sudden death in the FXS population, investigations of persons diagnosed with both epilepsy and severe learning disorders revealed a risk for SUDEP three times the risk of patients with epilepsy alone (Nashef, Fish, Garner, Sander, & Shorvon, 1995) suggesting that those with both FXS and epilepsy also have an increased risk of sudden death. There is also mixed evidence to suggest that sleep apnea, especially obstructive sleep apnea, in the FXS population is elevated, although it has been proposed that facial abnormalities which accompany FXS may contribute to this risk with further reports suggesting the actual risk

is equal to the general population (Hagerman & Hagerman, 2002; Hersh & Saul, 2001; Juncos et al., 2011; Kidd et al., 2014; Tirosh & Borochowitz, 1992;). More recent evidence highlights this increased risk of apnea as being specific to persons diagnosed with FXS and ataxia syndrome—a movement disorder that is seen in a subgroup of FXS patients (Hamlin, Liu, Nguyen, Tassone, Zhang, & Hagerman, 2011). Interestingly, an increased incidence of apnea raises the distinct possibility of hypoxia-related Purkinje cell loss or damage, and there is evidence to support this relationship. Post-mortem investigations of persons with FXS who died suddenly have revealed that these deaths were likely the result of cardiorespiratory failure, and were associated with an increased incidence of cerebellar Purkinje cell loss and vermal atrophy (Greco et al., 2011; Sabaratnam, 2000). Whether the *Fmr1* mouse experiences these apneic episodes remains to be investigated.

Lurcher. The *Lurcher* mouse has been used extensively for research on olivoponto cerebellar degeneration for over half a century. Litters of these mice are comprised of wildtype mice, mutant mice heterozygous for the spontaneous mutation of the glutamate receptor ionotropic delta 2 (GRID2) gene, and homozygous non-viable mice which die shortly after birth (Vogel, Caston, Yuzaki, Mariani, 2005). The cerebellum of the mutant mouse develops normally following birth but then exhibits the spontaneous GRID2 mutation which results in apoptosis of the cerebellar Purkinje cells beginning around postnatal day (PND) 8 and culminating with nearly 100% loss by PND 28 (Caddy & Biscoe, 1979; Zuo et al., 1997). Thus this mouse is a model of developmental cerebellar neuropathology that has allowed researchers to identify the specific roles of Purkinje cells.

Investigation into the role of the cerebellum in modulating respiration events has revealed that that loss of cerebellar Purkinje cells in the *Lurcher* mutant is without effect on eupneic breathing, but causes deficits in initiating and maintaining appropriate respiratory response patterns to environmental challenges. The *Lurcher* mutant mouse, when exposed to minimally challenging levels of CO_2 (2% and 4% CO_2, 21% O_2, N_2 on balance), was slow to initiate compensatory increases in minute ventilation which persisted when re-introduced into normal room air conditions (0% CO_2, 21% O_2, 79% N_2). This abnormal breathing pattern during recovery consisted of large sporadic gasps (large TVs) and extended apneic-like end-expiratory pause periods in the mutant mice, but not the wildtypes (Calton et al., 2014; Calton et al., 2016).

***mdx*.** The *mdx* mouse, another unique animal model of developmental cerebellar Purkinje cell pathology, is a model of human Duchenne muscular dystrophy (DMD), a progressive disease of muscle deterioration which affects approximately 1 in 7,250 human males (Romitti et al., 2015). The X-linked mutant *mdx* mouse exhibits a spontaneous point-mutation in the DMD gene resulting in a loss of expression of the protein dystrophin (Campbell, 1995; Sicinski et al., 1989). Loss of muscular dystrophin in humans results in progressive myopathy and eventual early death due to cardiorespiratory failure as a result of diaphragm deterioration (Gardner-Medwin & Sharpies, 1989). In the *mdx* mouse this myopathology is less pronounced than in the human condition with minimal reductions in life span, making this model more appropriate for studying the effects of the central rather than peripheral loss of dystrophin (Beastrom et al., 2011; Chamberlain, Metzger, Reyes, Townsend, & Faulkner, 2007). However, the precise role of dystrophin in the CNS remains controversial.

Within the CNS, dystrophin is most concentrated in the cerebellum and co-localized postsynaptically to cerebellar Purkinje cells (Lidov, Byers, Watkins & Kunkle, 1990). Further, the lack of dystrophin in the cerebellum results in decreased Purkinje cell size, and reduced synaptic placticity as long-term depression of the Purkinje cells is inhibited (Anderson, Head, & Morely, 2004). This reduction in synaptic plasticity is thought to contribute to the cognitive deficits observed in boys with DMD, with nearly 30% classified as intellectually impaired with a mean intelligence quotient one standard deviation below the corresponding normally developing population (Anderson, Head, Rae, & Morely, 2002; Cotton, Voudouris, & Greenwood, 2001). Disordered breathing within the DMD population, especially hypercapnic hypoventilation, is thought to be the sole result of the diaphragm musculature deficit and has been shown to increase in incidence along with increased muscular dystrophy (Ragette, Mellies, Schwake, Voit, & Teschler, 2002). Mild diaphragmatic inflammation has been reported as early as three months of age in *mdx* mice with accompanying minor decreases in minute ventilation compared to controls (Huang et al., 2011). It remains to be examined, however, if patterns of disordered breathing occur in *mdx* mice prior to measurable respiratory musculature degeneration.

Purpose and Hypotheses

Experiment 1. The purpose of this study is to examine compensatory respiratory responses in multiple models of cerebellar neuropathology with differing levels of Purkinje cell loss or dysfunction to examine the specific significance of the Purkinje cells. Given that mutant *Lurcher* mice, with 100% global Purkinje cell loss exhibit significant respiratory response and recovery impairment during and following relatively

minimally challenging levels of O_2 and CO_2 exposure (Calton et al., 2014), it seemed reasonable that the next area of investigation should address the question of whether less severe forms of cerebellar Purkinje cell neuropathology would elicit similar effects, thus revealing the cerebellar Purkinje cells as a particularly key component of compensatory respiratory responses. Thus, it was hypothesized that the contribution of the cerebellar Purkinje cells to compensatory respiratory breathing patterns is so significant that the level of loss or dysfunction would be irrelevant—no differences between cerebellar mutant strains would emerge. It was further hypothesized that mutant *Fmr1* and *mdx* mice would exhibit similar patterns of respiratory compensatory deficits as mutant *Lurcher* mice when environmentally challenged with decreased levels of O_2, increased levels of CO_2, or both, when compared to their wildtype littermates. Specifically, within the three groups of cerebellar mutant mice (*Fmr1*, *Lurcher*, and *mdx*), it was expected that the mutants would respond to hypercapnic and hypoxic conditions less robustly than their wildtype littermates and this would be evidenced by smaller increases in TV or *f* or both during exposure. Further, the mutant genotypes within the three groups of mice would show significant impairment in the ability to recover from hypercapnic and hypoxic conditions in comparison to their wildtype littermates, which would be evidenced by a diminished ability to reestablish baseline TV and *f* values in comparison to the respiratory patterns of their wildtype counterparts. Finally, when breathing room air at baseline, no significant differences between mutant and wildtype mice were predicted.

Experiment 2. Following the finding of differences in breath frequency during recovery in experiment 1, the purpose of this study was to examine, in depth, two

components of breath frequency (TV and EEP) to pinpoint what may be driving the observed differences. Thus the first hypothesis was, taking into account weight in accordance with experiment 1, at baseline (room air) no differences between strain and genotype would occur in either TV or EEP—the animals would have equivalent depths of breathing and would all pause for an equal period of time between breaths. During hypercapnic recovery conditions it was further hypothesized that differences in TV would not occur—all animals would exhibit a progressive increase in TV in accordance with increasingly challenging levels of hypercapnic recovery. However, it was further hypothesized that during hypercapnic recovery conditions, differences observed in breath frequency from experiment 1 would be a direct result of EEP durations among genotypes. Specifically, mutant mice from each strain that exhibited reduced breath frequency in experiment 1 would present prolonged EEP times compared to their wildtype littermates.

Chapter 2: Experiment 1 Methodology

Animals

The experimental animals for this project consisted of 36 randomly selected litter-mate pairs (12 mutant and wildtype pairs for each of the three mouse strains: *Fmr1*, *Lurcher*, and *mdx*) that will were aged PND 60 at the onset of testing. A single pair was selected from each mating cage in order to avoid litter effects. Animals were bred and maintained in the Animal Care Facility located in the Department of Psychology at the University of Memphis. All animals were weaned at PND 25 +/- 3 days and sibling-housed in groups of 3-5 in ventilated polystyrene cages. Animals were continuously maintained in a temperature controlled environment (21±1ºC) on a 12:12 light-dark cycle (lights on at 0700) and given access to food and water *ad libitum*. Original breeders (*Fmr1*, #004624, *Fmr1* control #004828; *Lurcher*, #001046; *mdx*, #001801, *mdx* control #000476) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). It should be noted that each of these mutant mouse strains are on different genetic backgrounds (*Fmr1* = FVB, *Lurcher* = C57BL/6J, and *mdx* = C57BL/10J). While genetic background has been shown to have a profound influence on behavioral phenotype (Doetschman, 2009; Spencer et al., 2011), we additionally hypothesized that the effect of genetic background is much smaller than the effects of cerebellar neuropathology on stressed breathing. Thus, mice made on different genetic backgrounds were specifically selected for these experiments. Although using multiple mutant mouse strains that share a common genetic background is clearly a more popular approach with its own advantages (Doetschman, 2009), the only way to determine if a phenotype is robust with respect to genetic background is to compare across multiple backgrounds. 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.

Breeding

Fmr1. The breeding procedures for *Fmr1* mice consisted of two phases. The first phase entailed the mating of male mice hemizygous for the *Fmr1*^{tm1Cgr} targeted mutation (FVB.129P2-*Fmr1*$^{tm1Cgr/J}$) with wildtype control females (FVB.129P2-*Pde6b*$^{+}$ *Tyr*$^{c-ch}$/AntJ). This breeding strategy produced F2 litters composed of heterozygous females and wildtype males. Phase two consisted of mating F2 littermates to produce F3 litters of experimental mice that included hemizygous and wildtype males. Genotypes of all mice were confirmed at weaning with tail-snip polymerase chain reaction testing (PCR) through an outside source (Transnetyx, Cordova, TN, USA).

Lurcher. The inbreeding of *Lurcher* mice entailed the filial pairing of a non-ataxic female wildtype (WT; B6CBACa *A*$^{w-J}$/*A*-*Grid2*$^{+}$) with a mutant ataxic male heterozygous for the *Lurcher* spontaneous mutation (Lc/+; B6CBACa *A*$^{w-J}$/*A*-*Grid2*^{Lc}). This breeding strategy produced litters composed of both heterozygous Lc/+ and WT mice. The Lc/+ mouse expresses this heterozygous Grid2 mutation resulting in nearly 100% developmental cerebellar Purkinje cell loss by PND 28 and this mutation is phenotypically observable as early as PND 12 by ataxic gait, permitting the non-invasive differentiation of Lc/+ mice from their non-ataxic WT littermates (Zuo et al., 1997).

mdx. Breeding the *mdx* strain of mice also consisted of two phases. The first phase entailed mating original male mice hemizygous for the spontaneous X-chromosome linked muscular dystrophy mutation (C57BL/10ScSn-*Dmd*^{mdx}/ J) with

female controls (C57BL/10ScSnJ) to produce F2 litters of heterozygous females and wildtype males. Phase two consisted of mating F2 littermates to produce experimental F3 litters that consist of homozygous and wildtype males. Genotypes of all mice were confirmed with tail-snip PCR at weaning through an outside source (Transnetyx, Cordova, TN, USA).

Whole Body Plethysmography

Data were collected using a whole body plethysmography system (WBP; Emka Technologies, Falls Church, VA, USA) in accordance with previously established procedures (Calton et al., 2014). Briefly, animals were placed, unrestrained, into a cylindrical plexiglass chamber (volume ≈ 450ml) while the percentages of environmental O_2, CO_2, and N_2 were manipulated by an in-house program using iOX2 software (Emka Technologies; Falls Church, VA, USA). A transducer is mounted to the WBP which converted pressure differentials in the chamber into electrical signals that were then transmitted to and interpreted by the software. An outflow ventilation pump is connected to the WBP to ensure a constant removal of exhaled CO_2 at 0.8L/min in order to prevent fatal accumulation of this gas.

Procedures

Mice were weighed prior to placement in the chamber and their weights (g) recorded for analysis. 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 (Falls Church, NC; USA; 2013), three programs created in-house that have been previously published (Calton et al., 2014) were used to assess the subjects' respiratory responses at baseline (normal room air; 21%

O_2, 0% CO_2 and 79% N_2), and under conditions of hypercapnia and hypoxia. Mice were randomly assigned to either the hypercapnia or hypoxia condition on PND 60—an age at which cerebellar neuropathology has been observed in these strains of mice (Koekkoek et al., 2005; Snow, Anderson, & Fry, 2014; Zuo et al., 1997), and the second condition followed on PND 61. Each test day began with a 10-min habituation period, followed by a 4-min exposure to baseline (Room Air) period with respective exposure to conditions of hypercapnia or hypoxia to follow baseline.

Baseline. Following the 10-min habituation period, 4-min of baseline measurements began. The dependent variables (DVs) tidal volume (TV, mL) and breath frequency (*f*, breaths per minute; bpm) were recorded continuously under normal room air conditions.

Hypoxia. The entire hypoxia program was 52 min in duration and consisted of one beginning baseline (4-min) measurement as described above followed by four sequential reductions in O_2 (19%, 17%, 15%, 13% O_2, N_2 on balance) with a return to normal room air between each successive challenge to minimize discomfort of the animals. Each of the four challenges consisted of a 2-min fill period (the amount of time necessary for the mass flow controllers to receive and execute the command to change the gas content of the WBP and for the WBP to subsequently reach the desired experimental gas percentages). The two minute fill period was followed by a 4-min exposure period. Following the final challenge (13% O_2) and subsequent return to room air, the mice were removed from the chamber.

Hypercapnia. The hypercapnia program followed the same timeline as the hypoxia program with the exception that following the baseline period, O_2 remained

constant at 21% and CO_2 was successively increased (2%, 4%, 6%, 8% CO_2, N_2 balance). Again, following the final challenge (8% CO_2) and the return to room air, the mice were removed from the chamber.

Variables and Data Analyses

The DVs in all conditions were TV and *f*. Breath sampling parameters for tidal volume (the volume of air inhaled in one breath) and breath frequency were set at approximately twice the normal, eupneic levels for mice to account for increased levels of minute ventilation across the exposure programs (typical TV < 0.20 mL; typical *f* < 500 bpm; Crosfill & Widdicombe, 1961; Lorenz, 2002). Breaths that met the sampling criteria were automatically averaged by the software in 10-s blocks across the duration of the 52-minute long experiment. Breaths that did not meet the sampling criteria were removed by the program automatically as movement artifacts.

Weights. Weights for the mice were recorded and analyzed with Strain (*Fmr1*, *Lurcher*, and mdx) and Genotype (mutant and wildtype) as the between subjects factors. Thus a 3 (Strain) x 2 (Genotype) analysis of variance (ANOVA) was performed to identify any weight differences. If significant differences were found, then weight was included in all subsequent analyses as a covariate. Using weight as a covariate in these instances eliminated the possibility of this factor having an effect on the results as it has previously been shown that body weight is tightly correlated with minute ventilation (Crossfill & Widdicombe, 1961). Correlations between weight and TV were further performed to identify the direction of the relationship between these variables.

Baseline. Because differences in body weight observed, the baselines collected prior to the hypercapnia and hypoxia conditions were compared using repeated measures

analyses of covariance (RMANCOVA), with separate analyses for each of the two DVs. Strain and genotype again served as the between subjects factors, Time Block (24, ten-second time blocks) served as the within-subjects factor, and weight served as the covariate. Thus, each analysis was a 3 (Strain) x 2 (Genotype) x 24 (Time Block) mixed design.

Hypoxia and Hypercapnia. For each experimental program, separate RMANCOVA analyses for each DV were performed. Strain and Genotype served as the between-subjects factors. The within-subjects factors included four levels of Gas (Hypoxia 19%, 17%, 15%, 13% O_2, or Hypercapnia 2%, 4%, 6%, 8% CO_2). Each gas was subdivided into six, 2-minute Conditions (Fill, Exposure 1, Exposure 2, Refill, Recovery 1, Recovery 2). The 2-min conditions were each further subdivided into 12, 10-s Time Blocks in order to accurately track changes in the variables. Finally, weight served as the covariate. Thus, each omnibus analysis was a 3 (Strain) x 2 (Genotype) x 4 (Gas) x 6 (Condition) x 12 (Time Block) mixed design. Depending on the results of the Omnibus analyses, subsequent univariate analyses were used to analyze interaction effects.

Chapter 3: Experiment 2 Methodology

Animals

Hypercapnic data files for each of 36 experimental animals were run in accordance with the procedures described below. The experimental groups consisted of six randomly selected littermate pairs from experiment 1 from each of the three strains.

Procedures

Hypercapnic Data Files. The data files of each experimental mouse were reanalyzed with new parameters using Emka Technologies iOX2 software (Falls Church, NC; USA; 2013). The new paramaters eliminated the 10-second averaged time blocks and instead investigated each individual breath.

Weights. The weights of the randomly chosen mice was recorded for analysis as a covariate if necessary.

Baseline. The first 510 breaths from the new baseline file were used for analysis of the breath frequency components TV and EEP. In order to examine the individual components of breath frequency (TV and EEP) and to remove the confound of different breathing rates on these analyses an equal number of breaths (510) was chosen from all animals using previously described procedures (Calton et al., 2016).

Hypercapnic Recovery. The first 510 breaths from each of the four hypercapnic recovery conditions (2%, 4%, 6%, 8%) in the new data file were used for analysis. These breaths were extracted from the Recovery 1 and Recovery 2 periods described in experiment 1.

Variables and Data Analyses

The dependent variables in all conditions were TV and EEP. Breath sampling parameters remained the same as experiment 1. Breaths that met the sampling criteria were added to the data file for later analysis. The program automatically removed breaths that did not meet the sampling criteria as movement artifacts.

Weights. Weights were again recorded for analysis with Strain (*Fmr1*, *Lurcher*, and *mdx*) and Genotype (mutant and wildtype) as the between subjects factors. Thus a 3 (Strain) x 2 (Genotype) ANOVA was performed to identify any weight differences among the randomly selected pairs from the three different mutant mouse strains and determine the need for weight to be included in subsequent analyses as a covariate.

Baseline. Upon finding weight differences, the baseline collected prior to the hypercapnic conditions was examined using RMANCOVA with separate analyses for each of the two DVs. Strain and genotype served as the between subjects factors, while Breath (510) served as the within-subjects factor, and weights served as the covariate. Thus, each analysis was a 3 (Strain) x 2 (Genotype) x 510 (Breath) mixed design.

Hypercapnia Recovery. Two separate RMANCOVA analyses were performed for each DV (TV and EEP). Strain and Genotype again served as the between-subjects factors. The within-subjects factors included four levels of hypercapnia Recovery (from 2%, 4%, 6%, & 8% CO_2). Within each level of recovery the first 510 breaths were examined to identify moment-by-moment changes in breath frequency components. Weights again served as the covariate. Thus, each omnibus analysis was a 3 (Strain) x 2 (Genotype) x 4 (Recovery) x 510 (Breaths) mixed design. Depending on the results of the Omnibus analyses, additional univariate analyses were performed.

Chapter 4: Experiment 1 Results

Weights

The results of a one-way ANOVA revealed there were significant differences in weights between *Fmr1*, *Lurcher*, and *mdx* mice (Strain, F (2, 66) = 60.788, $p < .001$). Post-hoc analyses revealed *Lurcher* mice (M = 20.271 g, *SEM* .402) weighed significantly less than both *Fmr1* mice (M = 25.033 g, *SEM* .402) and *mdx* mice (M = 26.183 g, *SEM* .402). Additionally, Pearson's r revealed a significant positive correlation between the weights of the animals and their depth of breathing, r (70) = .430, $p < .001$ (Figure 1). Thus, as body weight has been shown to be an important determinant of minute ventilation all subsequent analyses included weight as a covariate in order to remove the effect of weight as a factor in the results.

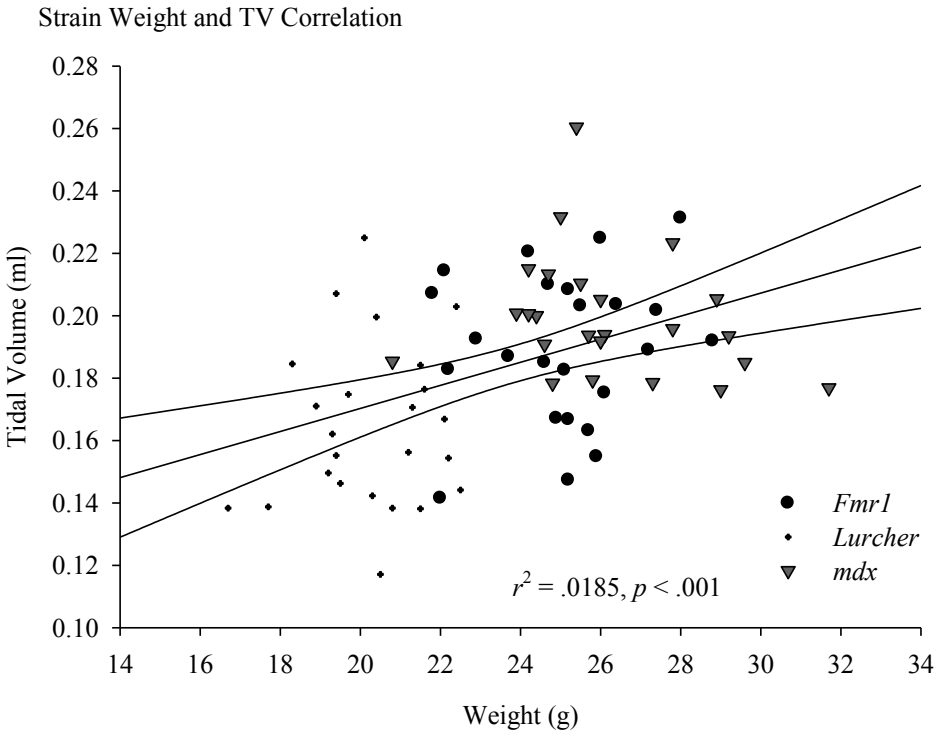

Figure 1. Correlation between strain weight and tidal volume. Body weights and corresponding averaged baseline TVs for each animal of each strain are plotted along the centered regression line flanked by 95% confidence intervals.

Baseline

Tidal Volume. During the baseline period, while breathing normal room air (21% O_2, 0% CO_2, 79% N_2), RMANCOVA revealed *Fmr1*, *Lurcher*, and *mdx* mice differed in their depth of breathing (Figure 2; Strain, F (2, 65) = 5.04, p = .009) with *Lurcher* mice demonstrating a much smaller TV (M = .165, SEM = .007) than *mdx* mice (M = .199, SEM = .006). However, the depth of breathing in mutant mice and wildtype mice was equivalent, (Genotype, F (1, 65) = 2.92, p = n.s.).

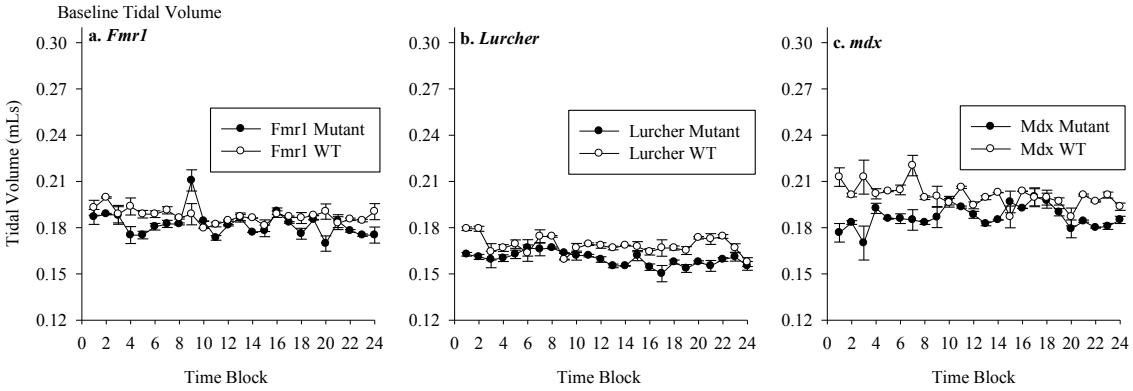

Figure 2. Baseline tidal volume for both genotypes of each strain across time blocks. Mean tidal volume (± *SEM*) for each of the 24, 10-second time blocks amongst all strains and genotypes during normal room air (21% O_2, 79%N_2, 0% CO_2).

Breath Frequency. A second RMANCOVA during the baseline period under normal, room air conditions, revealed that the frequency of breathing among *Fmr1*, *Lurcher*, and *mdx* mice, and among the mutant mice and wildtype mice was non-significantly different, (Figure 3; Strain, F (2, 65) = 2.19, p = n.s.; Genotype, F (1, 65) = .26, p = n.s.). Additionally, there was no interaction effect, (Strain x Genotype F (2, 65) = 1.13, p = n.s.).

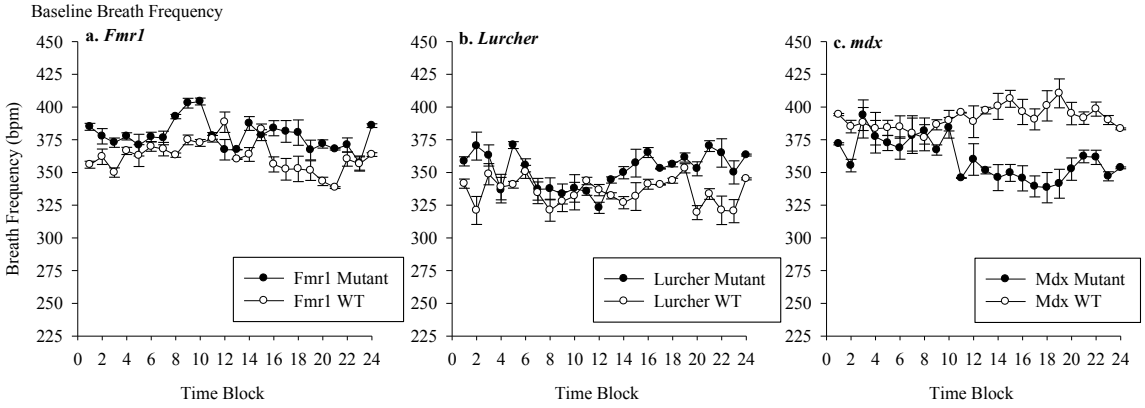

Figure 3. Baseline breath frequency for both genotypes in each of the three strains across time blocks. Mean breath frequency (± *SEM*) for each of the 24, 10-second time blocks amongst all strains and genotypes during normal room air (21% O_2, 79%N_2, 0% CO_2).

Hypoxia

Tidal Volume. The omnibus RMANCOVA revealed that exposure to decreased levels of atmospheric O_2 had the expected results; all mice displayed gradual reductions in tidal volume as O_2 decreased from 19% to 13% (Figure 4a; Gas F (3, 195) = 11.80, p = < .001). Additionally regardless of O_2 condition or time, *Lurcher* mice as a group breathed more shallowly than the *mdx* and *Fmr1* mice (Figure 4b; Strain F (2, 65) = 7.13, p < .01).

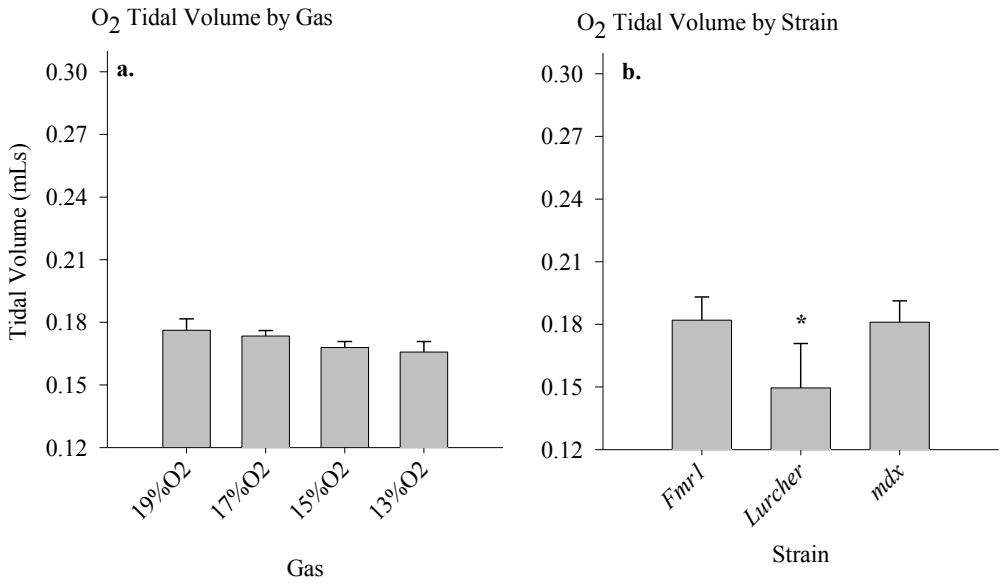

Figure 4. Main effects of gas and strain on tidal volume during hypoxia. Mean tidal volume (± *SEM*) for each of the hypoxic gas exposure periods amongst all strains and genotypes collapsed across conditions (**a**). Overall mean tidal volume (+/- *SEM*) for each mouse strain (**b**). ***** represents $p < .05$.

As shown in Figure 5 (a, b, c), the RMANCOVA also identified genotypic differences by strain in TV that were related to test condition (Fill, Exposure 1, Exposure 2, Refill, Recovery 1, and Recovery 2; Strain x Genotype x Condition F (10, 325) = 3.11, $p <$ .001). Regardless of gas exposure, the *mdx* mutants, showed larger reductions in TV than associated wildtype mice, that began to emerge during recovery 1 and intensified throughout recovery 2 (Figure 5c; Recovery 1, Genotype, F (1, 21) = 3.82, p = .06; Recovery 2, Genotype, F (1, 21) = 5.55, p = .028).

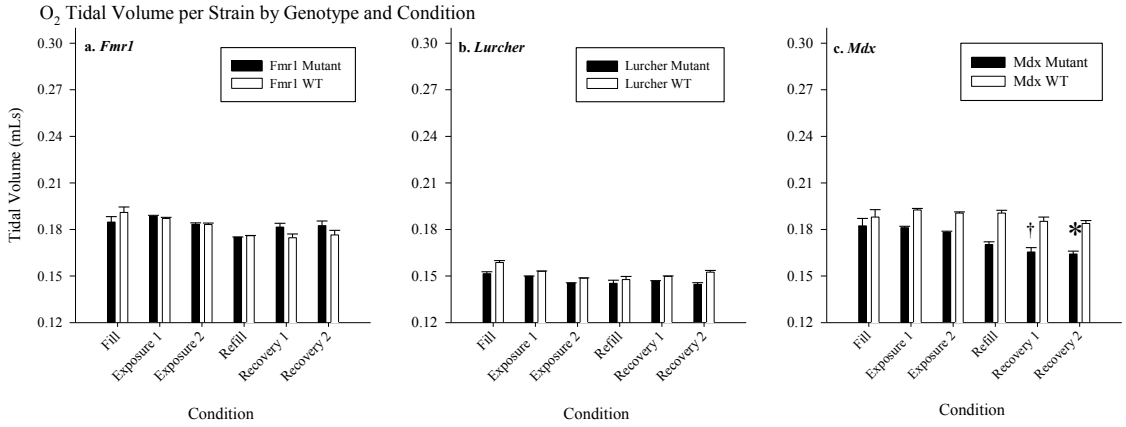

Figure 5. Recovery condition by strain by genotype effects on tidal volume during all hypoxic gas challenges. Mean tidal volume (± *SEM*) collapsed across all hypoxic gas exposures for *Fmr1* mutant and wildtype mice (**a**), *Lurcher* mutant and wildtype mice (**b**), and *mdx* mutant and wildtype mice (**c**). ***** represents $p < .05$, † represents $p < .06$

Breath Frequency. The omnibus RMANCOVA revealed two patterns of breath frequency reduction associated with changes in O_2 concentration and test condition. First, all mice decreased their breath frequency as O_2 concentration decreased (Figure 6a; Gas F (3, 195) = 53.37, p = < .001). Second, breath frequency declined significantly following exposure to reduced O_2 concentrations, during chamber refill and recovery to normal O_2 levels (Figure 6b; Condition F (5, 325) = 20.68, p = < .001).

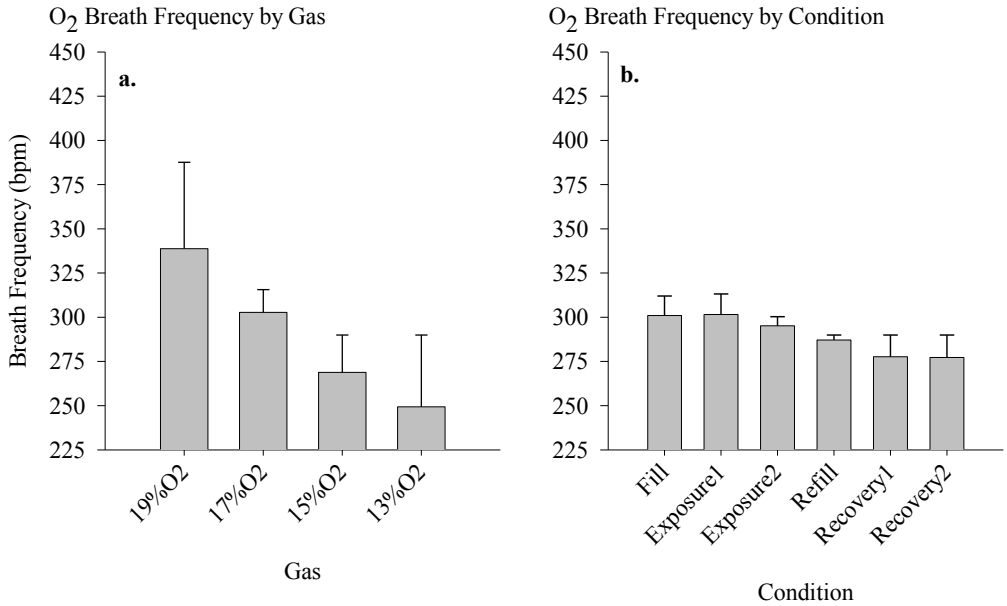

Figure 6. Main effect of gas and condition on breath frequency during hypoxic challenges. Overall mean breath frequency (± *SEM*) for each of the hypoxic gas exposures (**a**), and for each of the hypoxic conditions (**b**).

Further, when considered across the hypoxia program, breath frequency in the mutant and wildtype mice was equivalent (Data not shown; Genotype F (1, 65) = .05, p = n.s.). However, strain differences did emerge as *Fmr1* mice exhibited lower overall breath frequency than *Lurcher* and *mdx* mice (Figure 7; Strain F (2, 65) = 3.43, p = .04).

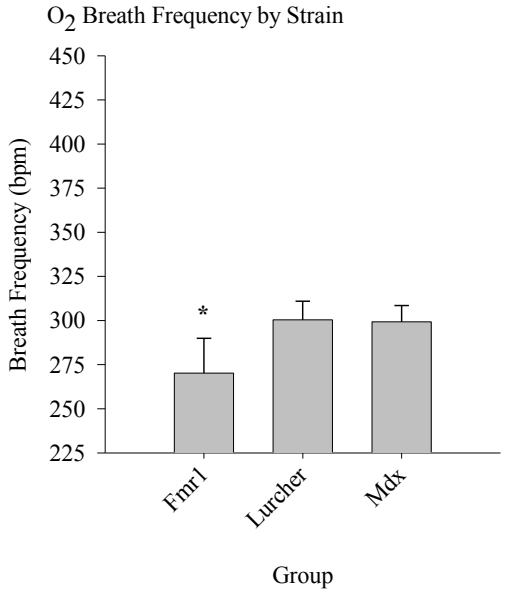

Figure 7. Main effect of group on breath frequency during hypoxic challenges. Overall mean breath frequency (± *SEM*) for each of the mouse strains across all hypoxic exposures and conditions. ***** represents $p < .05$

Hypercapnia

Tidal Volume. The omnibus RMANCOVA results indicated that when exposed to increasing levels of CO_2, all mice exhibited typical response patterns, increasing their depth of breathing as CO_2 concentration increased (Figure 8; Gas F (3, 195) = 64.65, p = < .001).

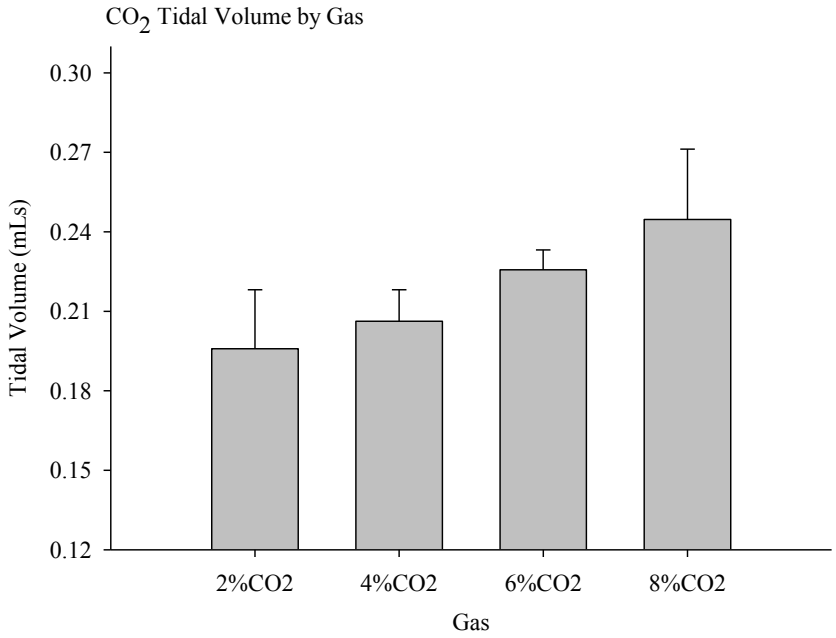

Figure 8. Main effect of gas on tidal volume during hypercapnic gas exposures. Overall mean tidal volume (± *SEM*) for all strains and genotypes.

Further, when considered across conditions all mice showed typical curvilinear patterns of tidal volume response respective to condition (Figure 9).

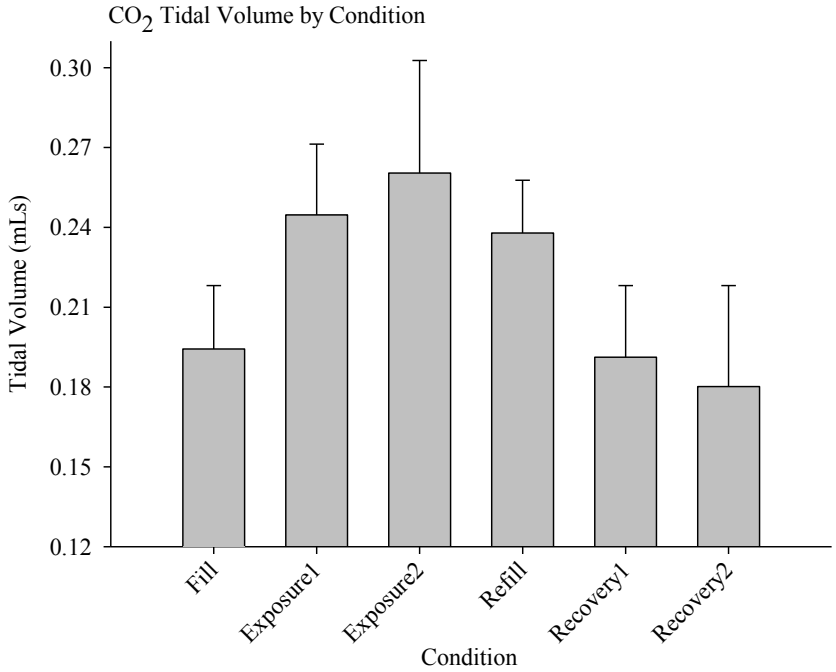

Figure 9. Main effect of condition on tidal volume during hypercapnia. Overall mean tidal volume (± *SEM*) for each of the six conditions regardless of gas, strain, or genotype.

In all mice and genotypes, the depth of breathing increased from the fill period to the exposure periods and then gradually decreased as they transitioned from the refill period and return to room air, to the final [recovery 2] condition (Figure 10; Condition F (5, 325) = 169.95, p = < .001).

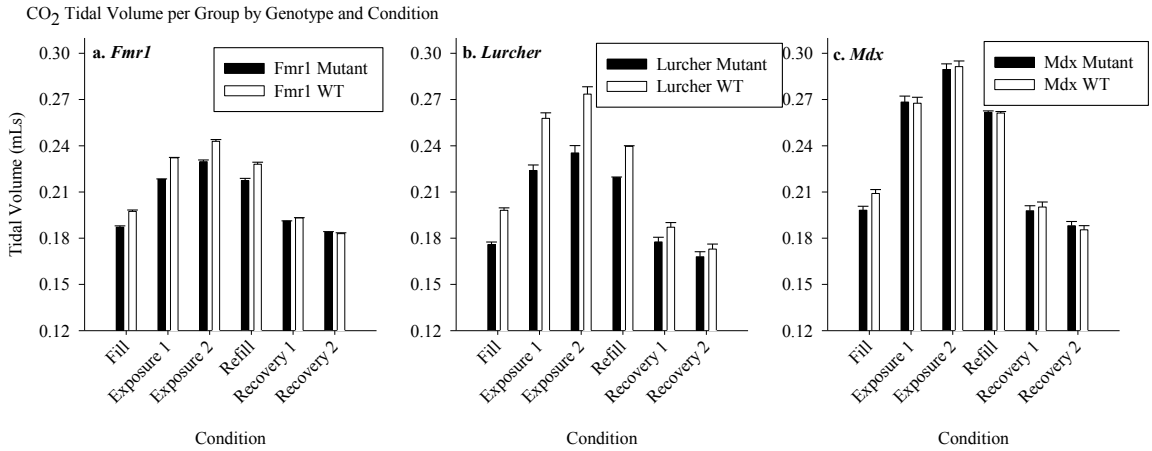

Figure 10. Mean tidal volume (± *SEM*) for each strain and each genotype across all of the conditions.

Breath Frequency. A final omnibus RMANCOVA revealed a typical compensatory pattern of breath frequency which decreased amongst all of the mice as CO_2 concentration increased (Figure 11a; Gas F (3, 195) = 21.93, p = < .001). All mice also exhibited a typical curvilinear pattern of breath frequency response among the six conditions. In all mice breath frequency increased from the fill period to exposure periods, and then decreased following exposure through the final recovery period (Figure 11b, Condition F (5, 325) = 33.94, p = < .001; Strain F (2, 65) = .32, p = n.s.).

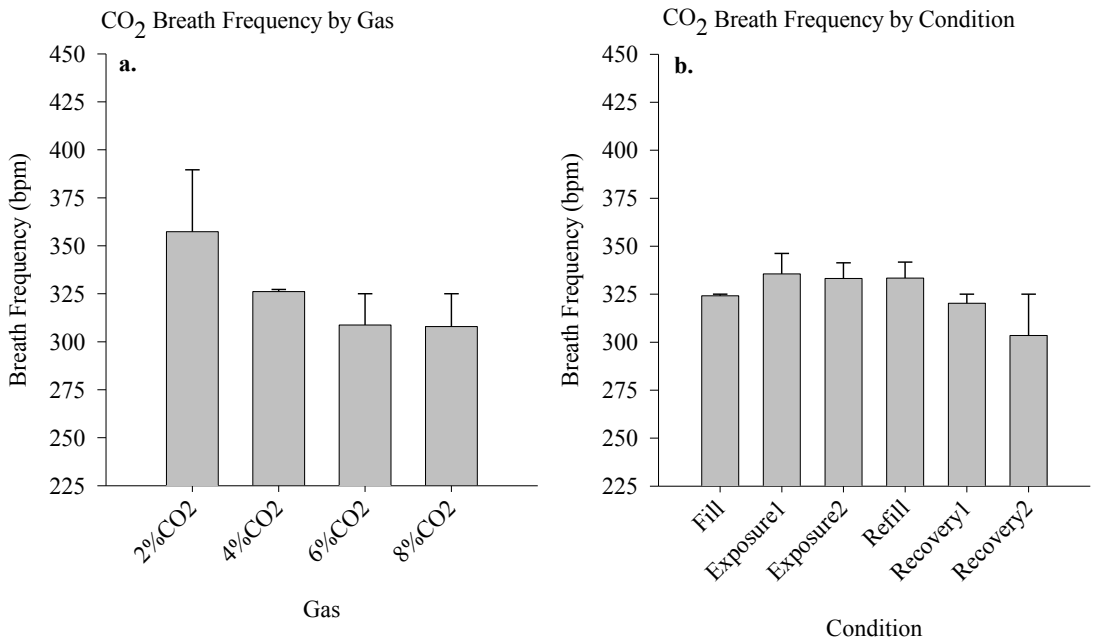

Figure 11. Main effect of gas and condition on breath frequency during hypercapnia. Overall mean breath frequency (± *SEM*) for each of the four hypercapnic gasses (**a**) and for each of the six conditions (**b**).

The omnibus RMANCOVA also revealed significant genotypic differences that occurred regardless of strain and in response to all changes in CO_2 level. All mutant mice exhibited reduced breath frequencies across the hypercapnia program compared to wildtype mice (Genotype F (1, 65) = 4.45, p = .04). As shown in Figure 12 (a, b, c) dependent on the condition, the breath frequency between the genotypes differed significantly by strain (Strain x Genotype x Condition F (10, 330) = 2.21, p = .017). Specifically, during the fill period *Lurcher* and *mdx* mutant mice were slower to respond to increasing levels of CO_2 compared to their wildtype littermates (Fill, *Lurcher* Genotype F (1, 21) = 6.39, p = .02, Figure 12b; *mdx* Genotype x time F (11, 242) = 1.79, p = .06, Figure 12c). During exposure 1 and exposure 2, mutant and wildtype mice in all groups had equivalent breath frequency. *Lurcher* mutants were the first to fail to maintain this increased level of breath frequency, beginning during refill to normal room air

(Refill, *Lurcher* Genotype F (1, 21) = 6.96, p = .02). Once the WBP achieved normal room air conditions, deficits in breath frequency occurred in all mutant mice. Thus, mutant *Fmr1*, *Lurcher* and *mdx* mice all had significantly reduced breath frequencies compared to their respective wildtype littermates during recovery 1 (Recovery 1, Genotype F (1, 65) = 5.45, p = .023) and this deficit diminished throughout recovery 2 (Recovery 2, Genotype F (1, 65) = 3.89, p = .053).

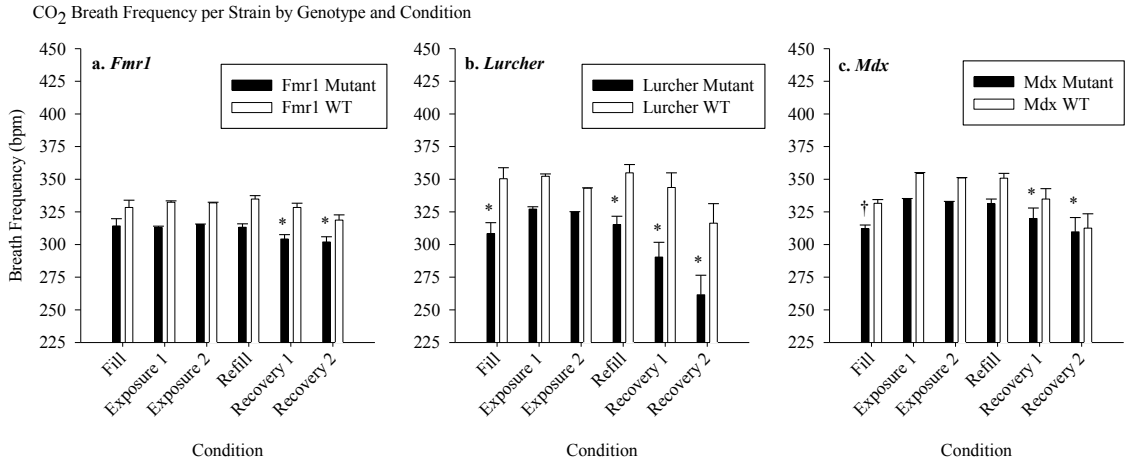

Figure 12. Effect of strain, genotype, and condition on breath frequency across hypercapnia. Mean breath frequency (± *SEM*) for each hypercapnic condition for *Fmr1* mice (**a**), *Lurcher* mice (**b**), and *mdx* mice (**c**). ***** represents $p < .05$, † represents $p < .06$.

Simple main effects tests revealed that across the two recovery conditions, *Lurcher* mutants were the most impaired. *Lurcher* mutants had the largest decreases in breath frequency compared to *Fmr1* and *mdx* mutants (Figure 13, Strain x Condition F (2, 33) = 6.03, p = .01).

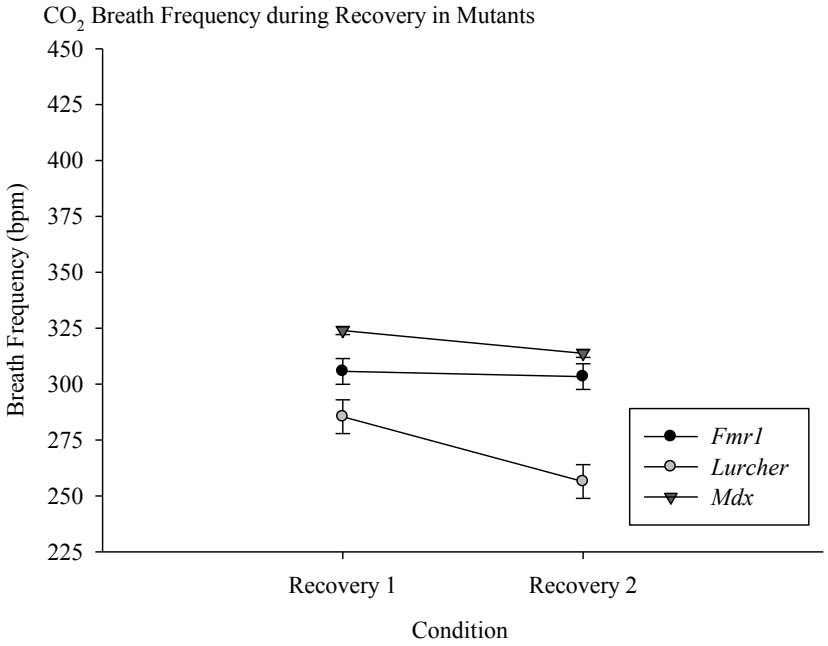

Figure 13. Mean breath frequency (± *SEM*) for mutants of each strain during recovery 1 and recovery 2.

Chapter 5: Experiment 2 Results

Weights

The results of a 3 x 2 between subjects ANOVA revealed a significant main effect of strain, (Strain F (2, 30) = 37.06, $p < .001$). Specifically, *Lurcher* mice weighed significantly less (M = 20.40 g, SEM = .526) than *Fmr1* (M = 25.00 g, SEM = .526) and *mdx* mice (M = 26.558 g, SEM = .526). However there was no difference between genotypes, F (1, 30) = .787, p = .382, and there was no interaction between group and genotype, F (2, 30) = 1.607, p = .217. Thus, as body weight has been shown to be an important determinant of minute ventilation all subsequent analyses included weight as a covariate to remove the effect of weight as a factor in the results.

Baseline

TV. Omnibus analysis revealed the depth of breathing (TV) across the 510 breaths during normal room air was significantly higher in *mdx* mice (M = .207, SEM = .021) than *Fmr1* (M = .184, SEM = .002) and *Lurcher* mice (M = .166, SEM = .020), (Figure 14; Strain F (2, 29) = 3.63, p = .039). However, this difference did not extend between the genotypes, (Genotype F (1, 29) = 1.04, p = n.s.). Further, there was no interaction between group and genotype, (Group x Genotype F (2, 29) = 0.11, p = n.s.).

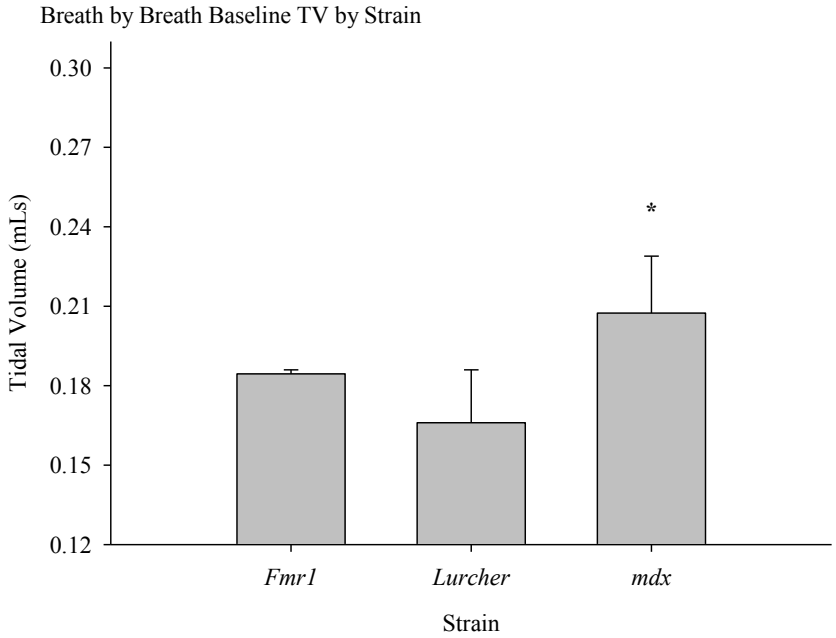

Figure 14. Main effect of strain on tidal volume during baseline at normal room air (21% O_2, 79% N_2, 0% CO_2) in experiment 2. Mean tidal volume (± *SEM*) for each mouse strain during baseline conditions across 510 breaths. ***** represents $p < .05$.

EEP. A second RMANCOVA revealed the time spent between exhalation and inhalation (EEP) did not differ by strain, (Figure 15; Strain F (2, 29) = .11, p = n.s.), or genotype, (Genotype F (1, 29) = .07, p = n.s.), nor was there an interaction between group and genotype, (Group x Genotype F (2, 29) = .85, p = n.s.).

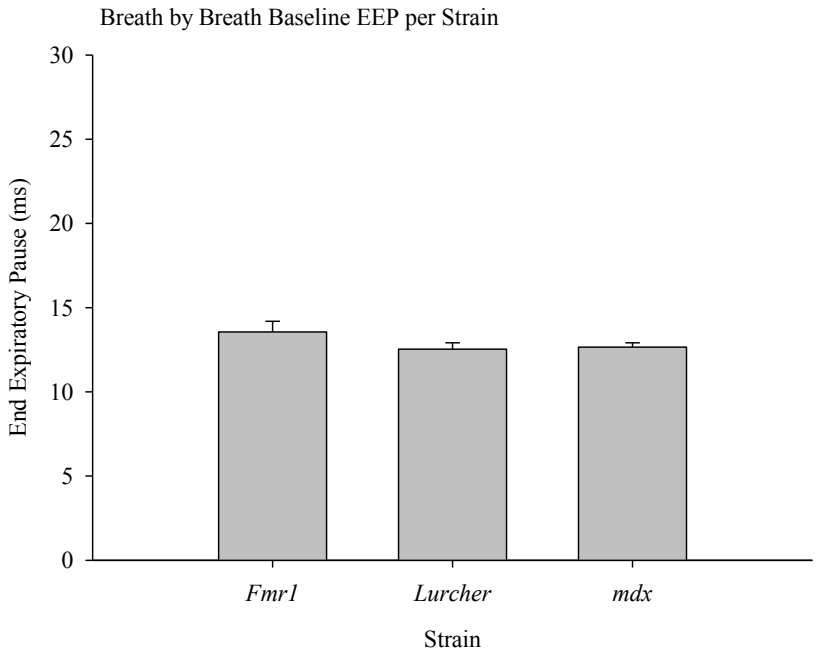

Figure 15. Main effect of strain on EEP during baseline at normal room air (21% O_2, 79% N_2, 0% CO_2) in experiment 2. Overall mean (± *SEM*) end expiratory pause (EEP) for each of the strains.

Hypercapnic Recovery

TV. Omnibus RMANCOVA revealed that during recovery all animals exhibited progressive increase in TV that corresponded to exposure to increasing concentrations of CO_2, (Figure 16; Recovery F (3, 90) = 4.73, p = .004).

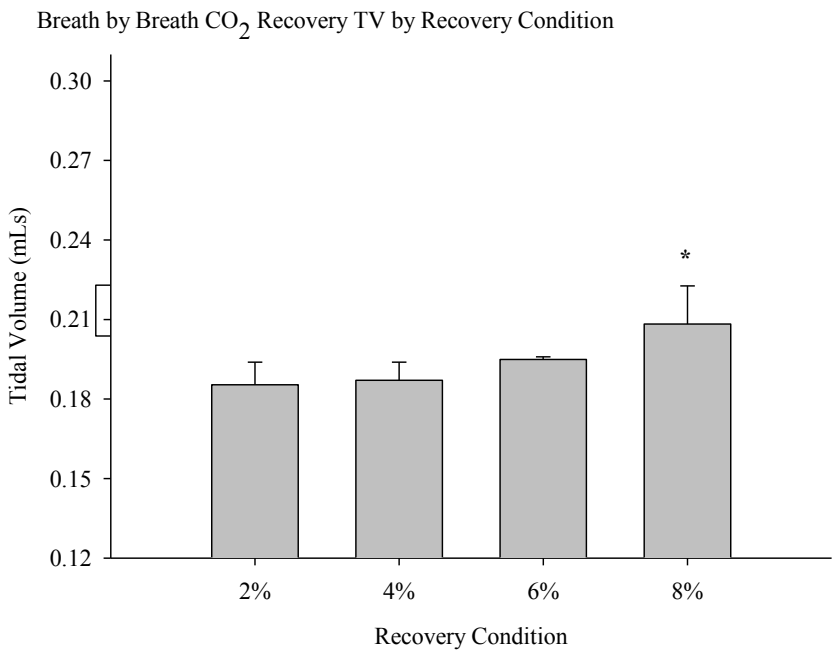

Figure 16. Main effect of recovery condition on tidal volume in experiment 2. Overall mean tidal volume (± *SEM*) for each of the four hypercapnic recovery conditions. * represents $p < .05$.

Further, *mdx* mice exhibited larger TVs overall ($M = .210$ ml, $SEM = .016$) than *Fmr1* ($M = .180$ ml, $SEM = .014$) and *Lurcher* mice ($M = .192$ ml, $SEM = .002$), (Figure 17; Strain $F(2, 29) = 4.04$, $p = .028$). However, these differences did not extend to genotype, (Genotype $F(1, 29) = .14$, $p =$ n.s.), nor was there an interaction between strain and genotype, (Strain x Genotype $F(2, 29) = .28$, $p =$ n.s.).

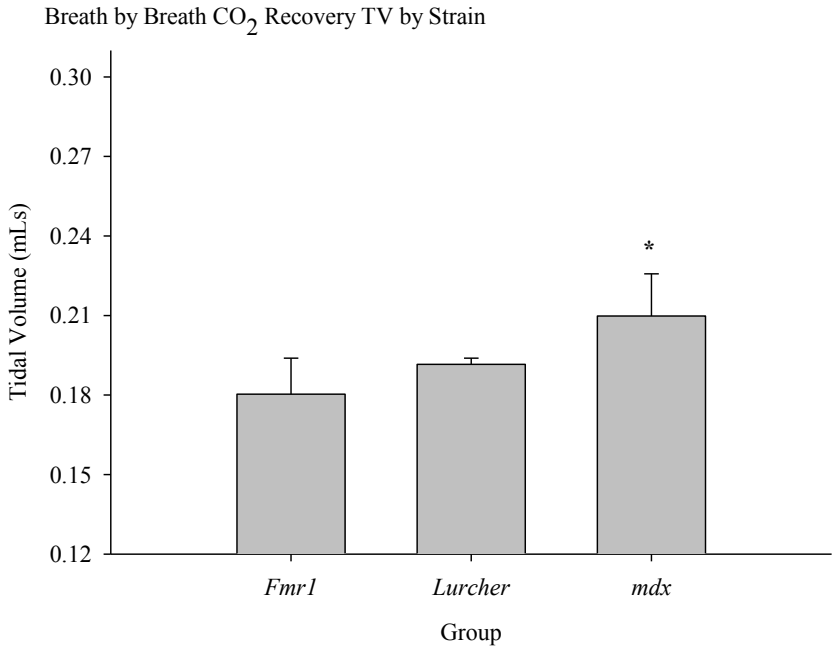

Figure 17. Main effect of strain on tidal volume in experiment 2. Overall mean tidal volume (± *SEM*) for each of the three mouse strains collapsed across all hypercapnic recovery conditions. ***** represents $p < .05$.

EEP. The omnibus RMANCOVA revealed all animals had significantly increased their end expiratory pause durations between breaths corresponding to recovery from increasingly challenging levels of hypercapnia, (Figure 18; Recovery F (3, 90) = 2.79, p = .045), and this difference peaked at 6% recovery (M = 22.718 ms, SEM = 2.317).

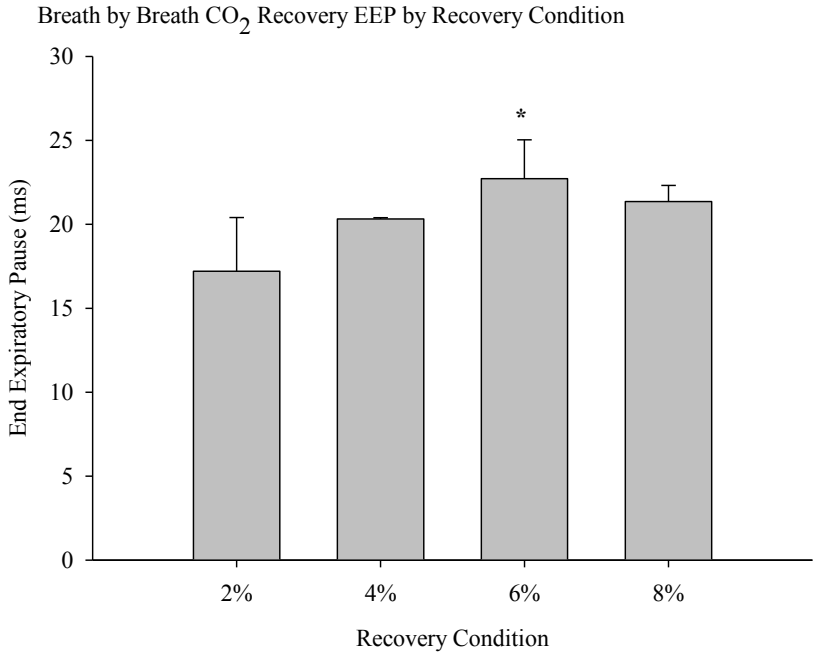

Figure 18. Main effect of recovery condition on EEP during recovery in experiment 2. Mean EEP (± *SEM*) time for all animals at each of the four hypercapnic recovery conditions. * represents $p < .05$.

Additionally, regardless of strain, there was no significant difference in the amount of time the animals paused between breaths (EEP), (Strain F (2, 29) = .05, p = n.s.). Interestingly, regardless of strain, genotype appeared to have an effect on the pause time, with mutants pausing for longer periods of time between breaths (M = 23.680 ms, SEM = .558) than wildtypes (M = 17.121 ms, SEM = .558), although these differences only approached significance, (Figure 19; Genotype F (1, 29) = 3.96, p = .056). Analysis further revealed there was no significant interaction between the strains of mice and the genotypes, (Strain x Genotype F (2, 29) = .08, p = .926)

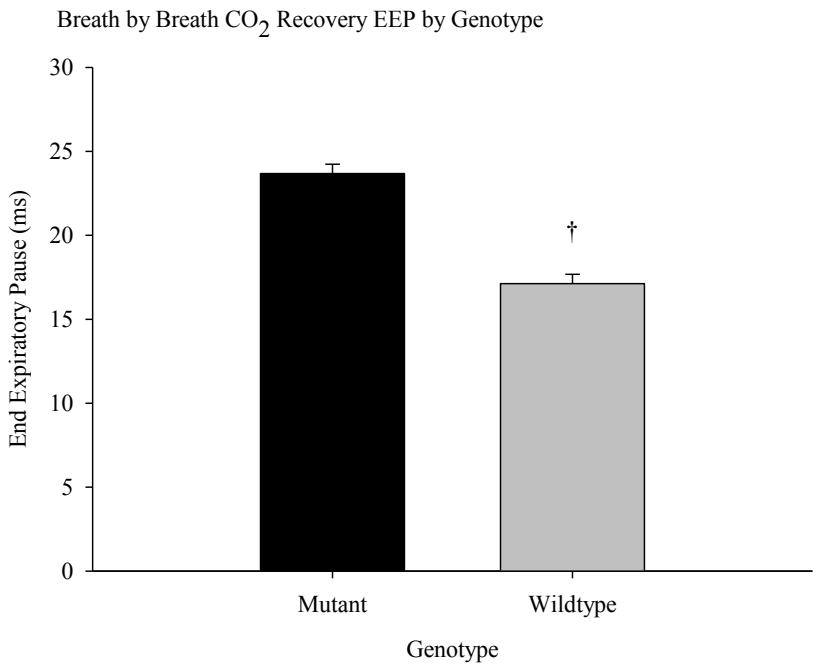

Figure 19. Effect of genotype on EEP during hypercapnic recovery in experiment 2. Overall mean EEP times (± *SEM*) for mutant and wildtype mice collapsed across all hypercapnic recovery conditions. † represents $p < .06$.

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Chapter 6: Discussion

Baseline

Differences between the animals' weights emerged and as expected revealed a positive relationship in that body weight was coupled to TV. These results are reflective of the typical relationship between body mass and respiratory function (Crossfill & Widdicombe, 1961; Kleinman & Radford, 1964). Once weight was controlled for, animals of all strains breathed at equivalent rates (*f*) and took an equal amount of time to reverse their breath direction (EEP). These results further suggest that neither the cerebellar neuropathology nor any potential muscle-related weakness in the genotypes of each of these strains has an effect on normoxic ventilation in PND 60-61 mice.

Even after weight had been used as a covariate, *Lurcher* mice still exhibited much smaller TVs than both *Fmr1* and *mdx* mice. However, as body weight is an indirect measure of assumed lung capacity, a more precise measure of lung capacity could be used as a covariate and would likely moderate these differences. Additionally, as the purpose of this investigation was to specifically identify the robustness of the effect of cerebellar neuropathological differences in breathing in multiple strains across multiple genetic backgrounds, this strain finding is ultimately unremarkable for the study at hand.

Hypoxia

The response to repeated exposures to challenging hypoxic conditions in mice of all strains and genotypes resulted in a small decrease in MV (Figs. 4a, 6a). These results are not entirely surprising as the response to mild hypoxia (O_2 levels > 10%) is far less robust than hypercapnia (Fong, 2010; Teppema & Dahan, 2010), and these same hypoxic response patterns were previously observed in *Lurcher* mice (Calton et al., 2014). The

overall decrease in MV across strains and conditions likely reflects the relatively brief time designated between successive hypoxic challenges, and suggests that mice were not given enough time to fully recover between these challenges.

Of unique interest were the response patterns of TV of *mdx* mutant mice across the hypoxic conditions (Figure 5c). While both genotypes of the *Fmr1* and *Lurcher* mice maintained equivalent breathing patterns across all of the conditions (from fill to recovery 2), the mutant *mdx* mice revealed a distinct inability to maintain their depth of breathing, with significant impairment by the time the animals reached recovery 2. That the *mdx* mutant mice TVs did not differ from the wildtype mice during normoxia or during the hypoxic fill and exposure conditions, and *mdx* mutant mice were able to attain and maintain TV levels during hypercapnia nearly double those observed in the hypoxic conditions (Figure 10), suggests this was not the result of an inability to achieve equivalent TVs.

It is possible that the significant reduction in TV following hypoxia may be indicative of fatigued diaphragm musculature in these animals as a result of dystrophin deficient myopathy (Figure 5c). While slow diaphragmatic degeneration begins around PND30 in these animals (Stedman et al., 1991), previous research has reported mixed evidence for a respiratory deficit prior to the first year of age in *mdx* mice. In one report, differences in MV between eight week old *mdx* mutant and wildtype animals were present during both baseline and hypoxic exposures (Burns, Edge, O'Malley, & O'Halloran, 2015). This possibility, however, appears unlikely as *mdx* mutants were fully capable of maintaining much larger TVs that were equivalent to wildtype mice when exposed to hypercapnic conditions (Figure 10c). Thus, a more parsimonious explanation

is that the impaired hypoxic recovery in *mdx* mice is related to peripheral chemoreceptor deficits attenuating the response patterns of the animals to hypoxia (Mosquiera, Baby, Lahiri, & Khurana, 2013).

It has been well established that respiratory response patterns to decreased environmental O_2 are far less robust than the response patterns to increased CO_2 and the control of these respiratory responses during development differs as well. Peripheral chemoreceptors are responsible for managing the hypoxic ventilatory response until the blood concentration of O_2 drops significantly enough to achieve hypoxemia—a point at which central chemoreceptors take over (Lagercrantz & Rohdin, 2008). In mice, it has been shown that moderate hypoxemia occurs after 10 minutes of exposure to 7.9% O_2 in 5 month old normal mice (Gonzalez, Kumar, Mulligan, Davis, & Saupe, 2004). Thus it is probable the hypoxic conditions presented to the animals in the current study were not challenging enough to create conditions for central chemoreception to take precedence over peripheral receptors, likely because they were exposed to relatively mild hypoxic conditions (19, 17, 15, and 13% O_2, N_2 balance) for a short period of time (4 minutes each). This possibility is supported by the fact that the other two strains did not reveal genotypic differences in minute ventilation, which if they had, would suggest central involvement of the fastigial nuclei (Figure 5). It has further been reported that in 5-7 month old *mdx* mice the diminished response to hypoxia is directly attributed to peripheral rather than central chemoreceptor deficits (Mosquiera et al., 2013). Additionally, that *mdx* O_2 blood levels are lower than controls during normal room air conditions (Mosquiera et al., 2013) suggests it may be even more difficult to challenge

these animals centrally with decreased O_2 levels, as chronically low arterial O_2 levels weakens the response to hypoxic stimuli (Severinghaus, Bainton, & Carcelen, 1966).

Hypercapnia

As anticipated, the most profound differences in MV emerged during the hypercapnia programs. First, as expected the TV and *f* response to hypercapnia in all animals followed a distinct curvilinear pattern, which accompanied the transitions across the six conditions (Figures 9, 11c). Animals increased their TV upon exposure to hypercapnia and reduced their TV during recovery—a characteristic response pattern (Moosavi et al., 2003; Sherwood, 1997). Further, the magnitude of change in TV increased as the level of hypercapnic challenge and recovery increased (2% TV < 4% TV < 6% TV < 8% TV; Figs 8, 16). Again, this was a typical response pattern as increased levels of hypercapnia elicit an increased response in MV in an effort to expel accumulating systemic CO_2. However a different and abnormal pattern of hypercapnic responses occurred for breath frequency that was apparent across genotype, regardless of strain.

While the wildtype animals in all strains maintained nearly unchanged breath frequency across all CO_2 exposures, mutant mice consistently had lower breath frequencies that were especially apparent during recovery (Figure 12). As mutants simultaneously maintained TV (Figure 10) at wildtype levels, and TV and *f* are components of minute ventilation, it is apparent that MV declined in all mutant mice. Breath by breath analysis further indicated that there were differences in EEPs between mutants and wildtype mice (Figure 19). Specifically, mutants had longer EEPs than wildtype animals which obviously contributed to the observed reductions in breath

frequency and MV in these animals. As the ability to increase MV is vital to effectively reducing blood levels of CO_2, it should be apparent that all mutants regardless of strain, showed a compromised ability to reduce the body burden of CO_2 and return to homeostasis (Edelman, Epstein, Lahiri, & Cherniack, 1973) and this vulnerability increases the level of concern for the likelihood of fatal CO_2 accumulation in the mutant mice across the three strains.

Relation to Previous Literature

Very little is known about disordered breathing patterns in the human FXS population and much of it is controversial. It has been reported that in 1,295 children with FXS or FXS carriers, persons with FXS reported an elevated incidence of sleep problems including excessive daytime sleepiness which is often used as an indicator of undiagnosed sleep apnea (Lavie, 1983; Kronk et al., 2010). Others who have directly investigated apnea in FXS patients have either reported that it occurs only in a subset of this population (FXS and ataxia syndrome) or that it only occurs in FXS patients with facial abnormalities and results in episodes of obstructive sleep apnea (Hagerman & Hagerman, 2002; Hamlin et al., 2011; Juncos et al., 2011; Kidd et al., 2014; Tirosh & Borochowitz, 1992). In the current study *Fmr1* mice exhibited patterns of disordered breathing (including extended EEPs) following hypercapnia, which alludes to the possibility that the associated human population may be vulnerable to apneic episodes only when faced with a relatively mild environmental hypercapnic stressor. This possibility deserves further investigation.

Multiple clinical investigations in patients with FXS have reported on two cerebellar deficits; hypoplasia of the cerebellar vermis (Hoeft et al., 2010; Mostofsky et

al., 1998) and cerebellar Purkinje cell loss (Greco et al., 2011). Pre-clinical studies using magnetic resonance imaging and immunohistochemical analysis of the deep cerebellar nuclei of 30 PND *Fmr1* mice revealed a significant reduction in the volume of the deep cerebellar nuclei, the main output of the cerebellum, compared to age- and sex-matched wildtype mice (Ellegood, Pacy, Hampson, Lerch, & Henkelman, 2010). Additionally, these authors noted a corresponding and significant reduction in the size of the fastigial nuclei of these mice.

The FN is a chemosensitive area of the cerebellum in humans and animals that modulates cardiorespiratory behavior when challenged and is especially responsive to hypercapnic conditions (Gozal et al., 1994; Lutherer et al., 1989; Xu & Frazier, 2002). Thus, it seems probable that a reduction in the volume of the FN would result in a reduction in chemosensitivity, limiting an organism's ability to respond to or recover from increases in blood levels of CO_2. Reductions in the size of the FN as well as all the DCN appear to be a direct consequence of Purkinje cell loss as both *Fmr1* mice and *Lurcher* mutants have this combined neuropathology (Heckroth, 1994). *Lurcher* mice have previously been shown to have a reduced ability to respond to and recover from hypercapnic conditions, exhibiting signs of post-sigh apneic-like episodes with extended EEP durations (Calton et al., 2014; Calton et al. 2016). These results were replicated in the current study. As *Fmr1* mutant mice also showed deficits in recovering from hypercapnia as effectively as their wildtype littermates, the current study highlights the importance of investigating challenged respiration, especially during hypercapnia, in persons with FXS. Specifically, based on the current results it would be expected that

FXS individuals may show increased incidences of apneic-like episodes (i.e. extended EEPs) after exposure to hypercapnia.

While extensive research has been performed on the dystrophin-deficient *mdx* mouse, there is scant literature on the progression of CNS modulated respiratory deficits that occur relative to well-described patterns of muscular [diaphragmatic] dystrophy in these animals. The few findings describing respiratory patterns in these animals have yielded mixed results. For example, a 20-minute, 10% O_2 exposure period averaged over one-minute intervals found attenuated MV in eight-week old *mdx* mice and controls during both baseline and hypoxic exposure (Burns et al., 2016). While research on seven month old *mdx* and control mice revealed no baseline (room air) differences in tidal volume or breath frequency, but a diminished response during exposure to 7% CO_2 in the mutant animals (Gosselin, Barkley, Spencer, McCormick & Farkas, 2003). Further, Gayraud et al. (2007) found no evidence for baseline or hypercapnic exposure differences in averaged breath frequency or tidal volume in five month old *mdx* mice. That the current findings further support evidence for no breathing differences in normoxia between *mdx* mice and wildtype mice by PND 60 despite mild diaphragm myopathy, it is quite possible these contradictory results may simply reflect the differing methodology used and additionally highlights the need for comparable testing strategies.

Although the precise role of dystrophin in the CNS remains largely unknown, it is widely believed that the learning disabilities associated with conditions such as Duchenne's muscular dystrophy are directly related to the central loss of dystrophin (Cyrulnik & Hinton, 2008; Kim, Wu, & Black, 1995; Uchino et al., 1994). Dystrophin has been found in the highest concentrations in the cerebellum, co-localized in cerebellar

Purkinje cells, which suggests a critical role of dystrophin in Purkinje cell function. In fact, evidence from dystrophin-deficient *mdx* mice has revealed evidence supporting a reduction of postsynaptic γ-aminobutyric acid ($GABA_A$) receptor clusters corresponding with a reduction in inhibitory input in Purkinje cells in the cerebella of these mice (Anderson et al., 2003). As reductions in Purkinje cell excitability would affect downstream DCN function including the FN, it is probable the observed reduced CO_2 sensitivity in *mdx* mice in the current study is an outcome related to dystrophin-mediated synaptic connectivity alterations.

Implications and Future Research

As disordered breathing patterns would put a person, regardless of age, at increased risk for ventilatory failure, and potentially subsequent sudden unexpected death, it appears necessary to extend investigation into populations that exhibit both cerebellar neuropathology and disordered breathing. Autism spectrum disorders are one such condition with an increased incidence of breathing disorders and frequently reported neuropathological changes to the cerebellum including Purkinje cell loss and DCN volume variations (Fatemi et al., 2012; Kemper & Bauman, 1998). Although several reports have described an increased risk of mortality in persons with ASD, especially in persons with ASD and epilepsy (Hirvikoski et al., 2016; Mouridson et al., 2008), the likelihood of sudden unexpected and / or unexplained death remains to be investigated in this population. Further, as ASD is frequently diagnosed in persons with other diagnosed neuropathological disorders, it would be advisable to expand the examination of sudden unexpected or unexplained death to multiple clinical conditions.

There are several neurodevelopmental disorders with high levels of comorbidity with ASD, cerebellar neuropathology, and disordered breathing including Fragile-X, Joubert, Prader-Willi, and Rett syndromes. These syndromes have documented cerebellar vermal agenesis or dysgenesis (Joubert—Joubert, Eisenring, Preston, & Andermann, 1969), cerebellar hypoplasia (Prader-Willi–Titomanlio et al., 2006; Rett—Schaefer et al., 1996) and cerebellar atrophy and Purkinje cell loss (FXS—Brunberg et al., 2002; Rett—Murakami, Courchesne, Haas, Press, & Yeung-Courchesne, 1992). It has been commonly reported that persons with these syndromes show an increased incidence of apnea during the day as well as a heightened incidence of sleep apnea (Joubert—Wolfe, Lakadamyali, & Mutlu, 2010; Prader-Willi—Festen et al., 2011; Holm, Cassidy, Whitman, & Butler, 1993; Rett—Weese-Mayer et al., 2006). It should also be noted that, in the syndromes that have been studied most extensively, there is an elevated comorbidity of seizures and epilepsy (FXS—Berry-Kravis, 2002; Prader-Willi—Vendrame et al., 2010; Zafeiriou, Ververi, & Vargiami, 2007; Rett—Dolce, Ben-Zeev, Naidu, & Kossoff, 2013) as well as evidence of increased prevalence of sudden death (FXS— Fryns, Moerman, Gilis, d'Espallier, & Berghe, 1988; Prader-Willi—Einfeld et al., 2006; Rett—Fyfe, Leonard, Dye, & Leonard, 1999). Thus, special emphasis should be placed on methodically examining the relationships among conditions presenting with ASD symptomology, cerebellar neuropathology (especially Purkinje cell loss or dysfunction and vermal abnormalities), disordered breathing, and sudden death.

Epilepsy is another challenging neurological condition for researchers that frequently presents with ASD symptomology and disordered breathing patterns as well as increased vulnerability to sudden unexpected death. Cerebellar neuropathology,

especially Purkinje cell loss, is common in persons with epilepsy and victims of SUDEP although the precise cause is controversial (Botez et al., 1988; Dam, 1987; Thom et al., 2015). While some researchers suggest the cause is related to repeated exposure to hypoxia following multiple grand-mal seizures (Dam et al., 2007; Spielmeyer, 1930), others suggest the cause may be related to AED use (Brostoff et al., 2008; Crooks, Mitchell, & Thom, 2000; McLain et al., 1980; Ney, Lantos, Barr, & Schaul, 1994). Regardless of the cause of the Purkinje cell loss in this population, however, the current study indicates that loss or even dysfunction of these cells results in disordered breathing patterns that may put someone at risk for sudden death. Thus, it is likely the cerebellar Purkinje cell loss observed in SUDEP victims may not simply be a benign effect, but rather may have contributed to the fatal event. Further, given substantial evidence of the relationship between the cerebellar Purkinje cells and the FN—a modulator of cardiorespiratory response patterns (Lutherer et al., 1989; Rector, Richard, & Harper, 2006; Xu & Frazier, 2002), and that treatments of epilepsy often include AEDs which induce or exacerbate cerebellar damage including atrophy and Purkinje cell loss (Brostoff et al., 2008; Crooks et al., 2000; Eldridge et al., 1983; Mclain et al., 1980), it can be concluded these treatments may be placing patients at further risk for sudden unexpected death and alternative approaches should be prioritized.

Dravet syndrome (DS) is an extreme form of myoclonic epilepsy that typically presents in infancy or early childhood and is associated with a significant rate of premature mortality and SUDEP (Genton, Velizarova, & Dravet, 2011; Kalume, 2013). Intellectual disabilities and ASD are present in up to 98% of children and adults with Dravet syndrome (Berkvens et al., 2015; Li et al., 2011). Although DS is generally drug

resistant, clinical symptom treatment often includes the use of multiple AEDs. Given the low response of DS to AEDs, the families of DS patients often resort to popular social treatments including adopting a ketogenic diet or using cannabinoid oil although scientific evidence of their efficacy is absent (Scichilone, Yarraguntla, Charalambides, Harney, & Butler, 2016). Clinical investigation has described recurrent cerebellar atrophy and Purkinje cell loss in a mixed population that included both living and deceased persons with epilepsy (including SUDEP victims) with or without DS (Catarino et al., 2011). Further, preclinical evidence of impaired cerebellar Purkinje cell firing is thought to cause ataxia associated with Dravet syndrome (Catterall, Kalume, & Oakley, 2010). Although direct evidence of disordered breathing in this subset of epilepsy patients remains to be described, it is commonly inferred that this group is particularly at risk for cardiorespiratory deficits—a key factor involved in SUDEP susceptibility (Kalume, 2013; Nashef, Hindocha, & Makoff, 2007). Considering the documented frequency of cerebellar neuropathology (specifically Purkinje cell loss) and the increased risk for SUDEP in the DS population, assumptions of disordered breathing patterns appear justified and should be verified in future investigations. Further it seems reasonable to conclude the pathologies reported in DS patients may be cyclical—repeated severe seizures result in hypoxic cerebellar Purkinje cell death, which would perpetuate intellectual disability or ASD symptoms and further increase the risk for disordered breathing. Disordered breathing patterns would, in sequence, increase the risk for hypoxic cerebellar neuronal death, and the cycle would repeat until the ultimate sudden demise of a challenged patient by cardiorespiratory failure. Thus, research in DS may be complicated and these relationships deserve further investigation.

Serotonin is another interesting component of breathing worth consideration. Serotonin, particularly important during development, exerts effects on cardiorespiratory behavior in a manner that is not entirely understood and neuropathological changes in the number or distribution of 5-HT containing neurons can directly influence this activity (Richter et al., 2003). The brainstems of SIDS victims consistently reveal 5-HT abnormalities including decreased 5-HT receptor binding in the medulla (Panigraphy et al., 2000). The *Lurcher* mouse, along with global cerebellar Purkinje cell loss, exhibits an extensive increase in 5-HT projections to the deep cerebellar nuclei, including the fastigial nuclei (LeMarec et al., 1998; Strazielle et al., 1996). As cerebellar Purkinje cell loss is the most commonly reported deficit in patients with ASD (Fatemi et al., 2012) and research reports ASD patients have pathological changes in platelet 5-HT which includes abnormally low levels during early development and abnormally high levels in adolescence and especially adulthood (Chugani, Muzik, Chakraborty, Mangner, & Chugani, 1998), investigation on central neuropathological 5-HT changes in this population should be pursued.

Medicinal treatment options for some of the symptoms of ASDs, FXS, and epilepsy includes the use of selective serotonin reuptake inhibitors (SSRIs), especially in early childhood (Albano, Cupello, Mainardi, Scarrone, & Favale, 2006; Aman, Lam, & Van Bourgondien, 2005; Berry-Kravis & Potanos, 2004) suggesting hypofunction of serotonin in these disorders. Interestingly, the use of SSRIs in clinical research has revealed these medications may be an effective method for modulating disordered breathing patterns including apnea (Kraiczi, Hedner, Dahlof, Ejnell, & Carlson, 1999). Further, a decreased hypercapnic ventilatory response has been reported in rats with

decreased cerebral 5-HT and SSRIs have been shown to augment this response (Hodges, Echert, Puissant, & Mouradian, 2013). Although how 5-HT dysfunction or dysregulation contributes to disordered breathing patterns remains unknown, it is evident this neurochemical system is significantly complex and future investigations should include examination of central serotonergic neuropathology in populations with disordered breathing. Further, given that persons with FXS, ASD, and Duchenne's exhibit apparent pathological serotonergic changes, 5-HT exerts a direct effect on cardiorespiratory behavior, and the current results suggest disordered breathing occurs during recovery from mild hypercapnic challenges in these groups, these complex relationships with the potential risk for sudden death should be considered.

It is important to address that although each of the aforementioned disorders may have additional neurological or physiological deficits, in the literature there is a clear pattern of relationship within these disorders with cerebellar neuropathology, autism spectrum disorder symptomology, disordered breathing, and sudden unexpected death incidence greater than the general population. As there are multiple categories of sudden death typically demarcated by developmental stage, special attention should be directed towards those who exhibit these apparently clustered risk factors regardless of age. Further, the consistent reporting of this tetra-factorial risk provides additional credence to the necessity of developing unified nationwide post-mortem sudden death protocols to further the knowledge base on these syndromes.

Conclusion

The purpose of this study was to investigate three components (TV, *f*, and EEP) of direct and indirect influence on minute ventilation under conditions of normoxia,

hypoxia, and hypercapnia in three mutant mouse strains that displayed a variety of developmental cerebellar neuropathologies in comparison to their wildtype littermates. The goal of these studies was to determine if these heterogeneous, cerebellar neuropathological differences contribute to stressed breathing, and thus contribute to an increased risk for sudden death. Throughout normoxia and hypoxic conditions mutant and wildtype mice maintained similar levels of minute ventilation and exhibited similar respiratory response patterns. However, in response to hypercapnic challenges, mutant mice of all strains revealed an impaired ability to appropriately recover by failing to maintain their breath frequency. This failure was a result of an increase in the pause time between breaths—a sign of an increased incidence of apnea. As apnea reduces an individual's ability to appropriately re-establish homeostatic blood O_2 and CO_2 levels, these results suggest not only substantial cerebellar Purkinje cell loss, but even mild dysfunction or dysregulation of the cerebellar Purkinje cells, may contribute to a failed respiratory response. Further, as cardiorespiratory failure is believed to be the cause of death in multiple sudden unexplained death events, it is possible the neuropathological findings reported in the cerebella of these victims did not occur as a result of the death, but rather preceded and contributed to these deaths. This possibility deserves further attention.

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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 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:

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

From:

☐ Your protocol is not renewed and the animals have been properly disposed of as described in your

IACUC Animal Research Protocol Revision Memorandum dated

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Prof. Guy Mittleman, Chair of the IACUC

_____

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