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Cerebellar Purkinje Cell Loss in Developing Lurcher Mice Influences Respiratory Compensatory Responses to Acute Hypercapnia and Hypoxia

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CEREBELLAR PURKINJE CELL LOSS IN DEVELOPING LURCHER MICE INFLUENCES RESPIRATORY COMPENSATORY RESPONSES TO ACUTE HYPERCAPNIA AND HYPOXIA

by

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Dedications

There are a number of people I would like to dedicate the completion of this thesis to, without whom I would have never achieved this goal.

First and foremost, I lovingly dedicate this thesis to my husband, John Calton, and our children, Jenna and Jonathon, who have supported me throughout this journey and have stood by me in my pursuits. They are my rocks and without them, this thesis would have never materialized.

Additionally, I dedicate this thesis to my mother, Dianna Crane, and my father, Grant Crane. Their unconditional love and support has proved priceless throughout my journey.

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Abstract

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Impaired responsivity to hypercapnia or hypoxia is commonly considered a mechanism of failure in Sudden Infant Death Syndrome (SIDS). We used a model of developmental cerebellar Purkinje cell loss to determine if such loss influenced compensatory ventilatory responses to hypercapnic and hypoxic challenges. Twenty-four Lurcher mutant mice and wildtype controls were sequentially exposed to 2% increases in CO_2 (0%-8%) or 2% reductions in O_2 (21%-13%) for four-minutes, with return to room air between each exposure. Whole-body plethysmography was used to continuously monitor tidal volume and breath frequency. Tidal volume increases to CO_2 elevation were reduced in Mutants, and slower to recover. Increased breathing rates to hypercapnia were also lower in Mutants, and were unable to be maintained in recovery, except for very high (8%) CO_2 levels. Less pronounced ventilatory responses emerged to hypoxia. Since cerebellar neuropathology appears in SIDS victims, developmental cerebellar neuropathology may contribute to SIDS vulnerability.

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CHAPTER 1: INTRODUCTION

Sudden Infant Death Syndrome

Sudden Infant Death Syndrome (SIDS) is characterized as the untimely, unexplained death during sleep of an otherwise healthy infant under the age of one even following a complete autopsy and environmental investigation (Krous, 2004). SIDS is a principal cause of death in children in developed countries and accounts for 22% of all infant deaths annually in the United States (Hauck & Tanabe, 2008; Malloy & MacDorman, 2005). Accordingly, the medical community has been working tirelessly for decades to identify, and potentially eradicate, the underlying mechanism(s) of SIDS, but to little avail. The early 1990s discovery of the relationship between SIDS and sleeping in the prone position led to the 1994 "Back to Sleep" campaign to educate care givers on the decreased risk for SIDS when infants were placed in the supine position to sleep (Willinger, Hoffman, & Hartford, 1994). In the advent of changing to a supine sleeping position the number of children that succumb to SIDS has decreased, however SIDS remains a leading cause of death in infants, suggesting that prone sleeping is only a partial contributor of vulnerability to this disorder (Hauck & Tanabe, 2008; Health & Development, 2001; Malloy & MacDorman, 2005; Moon, Horne, & Hauck, 2007; Trachtenberg, Haas, Kinney, Stanley, & Krous, 2012).

The Triple Risk Model

In 1970, a multifactorial approach towards the understanding of the underlying basis of SIDS was suggested and from then on forward, the theories surrounding SIDS research have been developed with this approach, proposing that SIDS is not the result of one factor, but rather several simultaneously occurring factors (Bergman, Beckwith, & Ray,

1970). According to the National Institutes of Health SIDS, these simultaneously occurring factors include having unknown brain abnormalities, being at a critical stage of development at the time of the fatal event, and being unable to properly compensate for, and/or recover from, exogenous respiratory stressors (Guntheroth & Spiers, 2002; Health & Development, 2001; Moon et al., 2007).

Unknown brain abnormalities. In the context of brain areas involved in SIDS it is necessary to understand that respiration has at least two prominent components. Eupneaic respiration refers to normal, relaxed breathing of normal air content while stressed breathing is essentially a compensatory response to changes in absorbed oxygen (O_2), carbon dioxide (CO_2), as well as blood pH levels (Sherwood, 1997). Although controversial, research suggests that there is limited overlap in brain areas involved in eupneaic and stressed breathing (McCrimmon & Alheid, 2004; Onimaru, Ikeda, & Kawakami, 2009). Specifically, a variety of brainstem networks have been implicated in the generation and maintenance of eupneaic breathing including the preBötzinger complex and the retrotrapazoid nucleus/parafacial respiratory group (Alheid & McCrimmon, 2009; Hilaire & Dutschmann, 2009; Spyer & Gourine, 2009). In histological examinations of the brains of SIDS victims, the brain stem does not appear abnormal, however there have been some subtle pathological findings both within and outside of the brainstem believed to be effects of the fatal SIDS event (Filiano & Kinney, 1994; Kinney & Thach, 2009). In 1992, Filiano addressed pathological findings of the arcuate nucleus in the brainstem in some SIDS victims and suggested the potential of furthering SIDS research by examining premudullary connections from other structures that may play a relatively specific role in breathing. One proposed location of inquiry

was the cerebellum, a structure that supports reflexive motor skills. It has been suggested that this structure may play a complementary role in respiration outside of eupnoea and instead induce apnea or limit the response of the brainstem to respiratory challenges such as increased carbon dioxide (CO_2) levels (hypercapnia) and decreased oxygen (O_2) levels (hypoxia; Haouzi, 2011; R. Harper, 2002). For example, complete cerebellectomy has no influence on eupneaic breathing, but markedly alters stressed breathing in response to reductions in O_2 and increased CO_2 (Xu & Frazier, 2000; Xu, Owen, & Frazier, 1995). The cerebellar influence on stressed breathing occurs via output through the deep cerebellar nuclei including the rostral fastigial, lateral, and interposed nuclei (FNr, LCN, IN) as ablation of these nuclei alters the response to increases in CO_2 (Xu & Frazier, 2002). Thus it is reasonable that the cerebellum importantly contributes to the compensation to, and recovery from, exogenous respiratory stressors (Harper, 2002; Harper & Kinney, 2010; Lu, Cao, Tokita, Heck, & Boughter, 2013; Xu & Frazier, 2002). Although neuropathology in both brainstem respiratory centers and the cerebellum has been reported in SIDS victims, it is unclear if this damage precedes the fatal event, or results from the acute hypoxemia that occurs immediately prior to a SIDS death (Filiano & Kinney, 1994; Haouzi, 2011; Harper, Richard, Henderson, Macey, & Macey, 2002; Kinney & Thach, 2009).

Critical period of vulnerability. Along with a proposed underlying brain abnormality, the model asserts that SIDS victims are in a critical stage of development. The timeframe in which infants are most vulnerable to SIDS is between birth until the age of one year, with a peak of susceptibility around three months—this timeframe coinciding with a very critical stage of brain growth, including divergent neural

synaptogenesis, pruning, and myelination in the growing infant (Health & Development, 2001; Tau & Peterson, 2010). During the first year of a life the infant brain increases to nearly 70% of its adult size, with the greatest amount of growth occurring in the cerebellum (Dekaban & Sadowsky, 1978; Knickmeyer et al., 2008; Tau & Peterson, 2010). Throughout a lifetime, the brain is highly sensitive to a large number of exogenous stressors, however due to this critical stage of growth the infant brain may have an exacerbated vulnerability to particular challenges during infancy (Tau & Peterson, 2010). In comparison to age-matched controls, delayed postnatal maturation of the cerebellar cortex has been demonstrated in SIDS victims, which could indicate a potential contributing factor accounting for cardiorespiratory failure (Cruz-Sanchez et al., 1997).

Exogenous stressors. The third assertion in the SIDS model is a failure to respond to, compensate for, and/or recover from, exogenous respiratory stressors. The two most frequently investigated exogenous stressors are hypercapnia and hypoxia, due to their relevance in cardiorespiratory failure and the likelihood that altered responsiveness to one, or both, may be involved in SIDS (Kinney & Thach, 2009). Thus, SIDS susceptibility has been determined using these stressors during sleep and wakefulness (Horne, Parslow, & Harding, 2005).

The normal content of air is 0% CO_2, 21% O_2, and 79% nitrogen (N_2). When challenged with an increased concentration of CO_2 or reduced O_2, the phenotypic respiratory response in both animals and humans is to increase the total volume of air breathed in one minute, which is known as minute ventilation (MV) to compensate for the change in rising blood CO_2 levels in an effort to maintain homeostasis. Minute

ventilation is further composed of two components: the volume of air inhaled in each breath (Tidal Volume, TV) and the number of breaths per minute, which is defined as breath frequency (*f*) (Knickmeyer et al., 2008; Moosavi et al., 2003; Sherwood, 1997). Both the responses to hypercapnia and hypoxia are indicators of stressed breathing. It should be noted however, that the degree of compensation to respiratory challenges in TV and *f* varies as a function of multiple factors including age, species and strain differences (Fong, 2010; Frappell, Lanthier, Baudinette, & Mortola, 1992; Tankersley, Fitzgerald, & Kleeberger, 1994). However, in infants and animals with particular brain abnormalities, such as cerebellar pathology, these normal responses may be inhibited (Harper & Kinney, 2010; Harper et al., 2002). It seems reasonable to assume cerebellar pathology may be another factor influencing variability in the degree of compensation to respiratory challenges in infants and animals (Harper, 2002; Harper & Kinney, 2010).

Purpose and Hypotheses

The purpose of this study was to determine, using a mouse model, the effects of developmental cerebellar neuropathology on the response to exogenous respiratory stressors. As noted previously, it is unclear if cerebellar neuropathology results from SIDS or is a component of vulnerability to this disorder. We used *Lurcher* mutant mice, which lose nearly all their cerebellar Purkinje cells during the first four weeks of life due to a spontaneous gain of function mutation in the delta-2 glutamate receptor gene (Grid2). These mutant mice were compared to their corresponding wildtype (control) littermates in their respiratory responses during conditions of hypercapnia and hypoxia (Caddy & Biscoe, 1979; Zuo et al., 1997). Purkinje cells comprise the sole output pathway of the cerebellar cortex and project through the FNr, LCN, and IN (Harvey & Napper, 1991;

Napper & Harvey, 1988). We hypothesized that *Lurcher* mutant mice with developmental cerebellar Purkinje cell loss would show deficits in responding to, and recovering from, acute challenges with CO_2 and O_2 compared to littermate, wildtype mice with normal numbers of cerebellar Purkinje cells.

CHAPTER 2: METHODOLOGY

Animals

Experimental subjects were bred and maintained in the Animal Care Facility located in the Department of Psychology at the University of Memphis. Mice were continuously maintained in a temperature controlled environment (21±1ºC) on a 12:12 light-dark cycle (lights on at 0700) and were given free access to food and water. Original Lurcher (#001046) breeders were purchased from The Jackson Laboratory (Bar Harbor, Maine). All experiments were approved by a local Institutional Animal Care and Use Committee and conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Breeding. The inbreeding of *Lurcher* mice entails the filial pairing of a non-ataxic female wildtype (WT; B6CBACa $A^{w\text{-}J}$/*A*-$Grid2^{+}$) with a mutant ataxic male heterozygous for the *Lurcher* spontaneous mutation (Lc/+; B6CBACa $A^{w\text{-}J}$/*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 post natal day (PND) 28 that is phenotypically observable by PND 21 as ataxic gait, which permits the non-invasive differentiation of Lc/+ mice from their non-ataxic WT littermates (Zuo 1997).

Animals were weaned at 21 PND +/- 6 days and sibling-housed in groups of 3-5 in ventilated polystyrene cages. The subjects for this experiment consisted of 24 male mice (12 Lc/+ and 12 WT) that were 60 PND of age at the start of testing and weigh 20g (+/- 3g).

Whole Body Plethysmography

Data were collected using a whole body plethysmography system (WBP; Emka Technologies; Falls Church, VA, USA). Animals were placed, unrestrained, into a cylindrical Plexiglas chamber (volume ≈ 450ml) Supplying gas to the chamber (at 1.0L/min) were three Allicat flow control modules, each calibrated for a separate gas (CO_2 and O_2: MC-200SCCM-D/10M, N_2: MC-1SLPM-D/10M, Tucson, AZ, USA). Gas mixture was controlled using an Allicat RS32 multidrop box (model BB9; Tucson, AZ, USA) that received input from a standard desktop computer via Emka Technologies iOX2 software (2013). A transducer was 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 (Emka Technologies; Falls Church, VA, USA) was connected to the WBP to ensure a constant removal of exhaled CO_2 at 0.8L/min in order to prevent accumulation of this gas.

Procedure

Mice were weighed prior to placement in the chamber and their weights recorded. Additionally, the experimental room temperature (21°C +/-1°C) and humidity (25% +/-10%) was monitored daily to ensure stability throughout the experiment. Using Emka Technologies iOX2 software, three separate programs 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 increased CO_2 (hypercapnia) and reduced O_2, (hypoxia). Mice were randomly assigned to either the hypercapnia or hypoxia condition on PND 60 and the second condition followed on PND 61. Each test day began with a 10-minute habituation period, followed by a 4 minute exposure to baseline (Room Air) period. Exposure to

conditions of hypercapnia or hypoxia followed baseline. Mice were exposed to continuously flowing room air at all times (0.8-1.0 L/m) during the habituation and baseline period.

Baseline. After the 10 minute habituation period, dependent variables including tidal volume (TV; ml) and breath frequency (*f*), were continuously recorded while the mice were exposed to room air for a total of four-minutes (21% O_2, 0% CO_2, 79% N). TV was defined as the volume of inhaled air in one breath and *f* was defined as the number of breaths per minute averaged every 10 seconds (both as received by the transducer and interpreted by the iOX2 software).

Hypercapnia. The entire hypercapnia program was 52 minutes long and consisted of one beginning baseline (4-minutes) measure as described above, followed by four sequential challenges where CO_2 was progressively increased from 2%, 4%, 6%, and 8%, (21% O_2, N_2 on balance). Each of the four CO_2 challenges consisted of a 2-minute chamber fill period (the time required for the WBP to achieve the desired gas percentages) followed by a four-minute exposure measurement. To minimize discomfort of the animals, between each challenge the program returned to room air (again including a two-minute chamber refill period and a four-minute recovery period). At the termination of the final CO_2 exposure (8%) and return to room air, the mouse was removed from the chamber.

Hypoxia. The same procedure was followed for hypoxia testing, with the exception that following the initial baseline period (4-minutes), O_2 was progressively reduced to 19%, 17%, 15%, and 13%, (0% CO_2, N_2 on balance). As above, reduced O_2 exposures

were separated by room air chamber refills and recovery periods. Thus, the duration of hypoxia testing was also 52 minutes.

Variables and Data Analysis

The dependent variables (DVs) in all conditions were TV and *f*. Both of these measures were averaged over 10-second intervals during baseline, hypercapnia and hypoxia testing.

Baseline. The two baselines collected prior to the hypercapnia and hypoxia conditions were compared using repeated measures analysis of variance (RMANOVA). Measures of TV and *f* were separately analyzed. In both analyses Genotype (Lc/+ and WT) served as the between subjects factor while Baseline (1 and 2) and Interval (twenty-four, 10-second intervals) served as the within subjects factors.

Hypercapnia and Hypoxia. For both conditions separate omnibus RMANOVAs were initially performed on TV and *f*. Genotype again served as the between subjects factor. Within subjects factors included four levels of gas exposure (Hypercapnia 2%, 4%, 6%, and 8% CO_2, or Hypoxia, 19%, 17%, 15%, and 13% O_2). Additionally, each gas exposure was subdivided into six, 2-minute time blocks such that the blocks corresponded to chamber fill (with the challenged amount of gas), gas exposure 1 and gas exposure 2 (which together comprise the four-minute exposure period), chamber refill (return to room air), and recovery 1 and recovery 2 (which together comprise the four-minute recovery period). The two-minute blocks were additionally divided into twelve, 10-second intervals in order to accurately track moment to moment changes in TV and *f*. Therefore the omnibus analysis was a 2 (Genotype) x 4 (Gas Exposure) x 6 (Time

Blocks) x 12 (10-second Interval) mixed design. Depending on the results of the omnibus RMANOVAs additional main effects tests were used to analyze interaction effects.

CHAPTER 3: RESULTS

Baseline

Tidal volume did not differ significantly across the baseline exposures to room air (Figure A1) shows this variable averaged over 10 second time intervals in Lc/+ and WT mice (Baseline, F (1, 22) = 1.15, p = 0.29). RMANOVA also indicated that TV in mice of both genotypes did not differ significantly (Genotype, F (1, 22) = 0.72, p = 0.41). Thus, average TV in Lc/+ and WT mice was respectively, 0.16 (SE = 0.01), 0.17 (SE = 0.01). TV remained constant between groups during exposure to room air (Group X Interval, F (23, 506) = 1.20, p = 0.24).

Breath frequency did not differ significantly across the two baselines and was averaged across exposures (Baseline, F (1, 22) = 3.64, p = 0.07). As shown in (Figure B1) breath frequency averaged 357.8 (SE = 10.14) in Lc/+ mice and 358.6 (SE = 10.14) in WT mice over baseline. RMANOVA confirmed that both genotypes had equivalent breath frequency (Group, F (1, 22) = 0.01, p = ns) which remained stable between groups over baseline intervals (Group X Interval, F (23, 506) = 1.25, p = 0.94).

Hypercapnia

Figure B1, panels a, b, c, & d illustrate the increase in tidal volume that occurred as a function of CO_2 increasing from 2% to 8% in Lc/+ and WT mice. Two patterns of change were apparent: first, during time blocks corresponding to increased CO_2 concentration (Exposure), TV consistently increased in both genotypes, and then declined when gas composition was returned to room air (Refill and Recovery; Time Block F (5, 110) = 25.97, p < .001). Secondly, as the concentration of CO_2 increased from 2 to 8%, there were progressive increases in the amplitude of TV (Gas Exposure X Time Block, F (15, 330) = 19.18, p < .001). Although Lc/+ mice consistently showed smaller increases

in TV during periods of increased CO_2 concentration the omnibus RMANOVA indicated that genotype related differences only approached significance (Genotype, F (1, 22) = 2.14, p = 0.16).

Figure B1, panels e, f, g, & h show the effects of increasing concentrations of CO_2 on breath frequency. Increasing levels of CO_2 produced small but significant reductions in breath frequency in both groups (Gas exposure, F (3,66) = 6.48, p < .001). Thus, in both genotypes at 2% CO_2, breath frequency averaged 344.38 (*SE* = 22.86) but was reduced to 316.50 (*SE* = 5.02), 309.59 (*SE* = 11.96) and 315.65 (*SE* = 5.88) as concentration increased respectively to 4, 6 and 8%. RMANOVA indicated that the two genotypes responded significantly differently at all CO_2 concentrations (Genotype, F (1, 22) = 7.85, p = 0.01) that varied as a function of time block (Genotype X Time Block, F (5, 110) = 2.34, p < 0.05). Simple main effects tests were used to specify the genotype-related differences at each time block. Results of these analyses are shown in figure C1. When considered across CO_2 concentrations Lc/+ animals consistently exhibited lower breath frequencies than WT controls during chamber fill, gas exposure 1, gas exposure 2, chamber refill (return to room air), recovery 1 and recovery 2. These genotypic differences were significant in all time blocks with the exception of the initial exposure to increased CO_2 (exposure 1). During this time block genotypic differences only approached significance (Exposure 1, F (1, 22) = 2.82, p = 0.11).

Hypoxia

Figure D1, panels a, b, c, & d shows the effects of decreasing concentrations of O_2 on tidal volume. As the concentration of O_2 decreased from 19 to 13%, there were progressive decreases in the amplitude of TV. Thus, for both genotypes TV averaged

0.155 ml (*SE* = 0.011) at 19% O_2 which progressively declined to 0.147 (*SE* = 0.004), 0.136 (*SE* = 0.007) and 0.135 (*SE* = 0.008) as O_2 concentration was reduced to 17, 15 and 13% (Gas Exposure F (3,66) = 26.77, $p < .001$). The omnibus RMANOVA indicated that when considered across reductions in O_2 concentration there were significant differences in TV between genotypes (Genotype X Time Block X Interval, F (55, 1210) = 1.61, $p = 0.003$). Follow up analysis revealed that during chamber fill, Lc/+ mice had significantly smaller tidal volumes than WT controls (Fill, F (11, 242) = 2.45, $p < 0.01$). As shown in Figure D1 this genotype-related difference occurred predominantly at O_2 concentrations of 13 and 15%.

Figure D1, panels e, f, g, & h indicates the effects of reductions in the concentration of O_2 on breath frequency. RMANOVA indicated that in both groups reductions in O_2 concentration caused a progressive decrease in average breath frequency from 326.15 (*SE* = 55.32) at 19% O_2 to 276.55 (*SE* = 5.73), 238.72 (*SE* = 32.11) and 241.88 (*SE* = 28.94) at respectively, 17, 15 and 13% O_2 (Gas Exposure, F (3, 66) = 36.82, $p < 0.001$). There were genotype-related differences in how Lc/+ and WT mice responded to reduced O_2 (Genotype X Gas Exposure, F (3, 66) = 2.91, $p = 0.04$; Genotype x Time Block X Interval, F (55, 1210) = 1.51, $p = 0.01$). Follow up analysis revealed a significant difference between genotypes at exposure to 17% O_2 (17%, F (1, 22) = 5.34, $p = 0.03$). As shown in figure D1, panel f Lc/+ mice had significantly lower breath frequencies during chamber refill and recovery following exposure to 17% O_2.

CHAPTER 4: DISCUSSION

Baseline

At baseline, Lc/+ mice and wildtype controls exhibited similar responses to normal room air in both TV and f. These results indicate that relaxed or eupnoeic breathing in these mice was equivalent. In agreement with previous results this finding provides additional support for the hypothesis that cerebellar damage is without influence on eupnoeic breathing (Xu & Frazier, 2002; Xu et al., 1995). Additionally, these results specifically demonstrate that developmental loss of cerebellar Purkinje cells is without significant influence on eupnoeic respiration.

Hypercapnia

Lc/+ mice exhibited significant deficits in compensating for increases in CO_2 in comparison to WT mice. Both genotypes showed a compensatory increase in TV during periods of exposure to increased CO_2 levels, and this increase in TV was magnified as CO_2 concentration increased from 2 to 8%. Although TV was consistently lower in Lc/+ mice, this difference only approached significance.

Breath frequency increased with exposure to increased concentrations of CO_2 and then declined upon re-exposure to room air in both genotypes. Considered together with the abovementioned changes in TV, this result indicates that both groups had a typical compensatory response to increases in CO_2 levels (Frappell et al., 1992; Knickmeyer et al., 2008; Moosavi et al., 2003; Sherwood, 1997; Tankersley, Fitzgerald, & Kleeberger, 1994). Importantly, when considered across all levels of increased CO_2, *f* was consistently lower in Lc/+ mice when compared to wildtype controls (Figure C1). This genotype-related difference in *f* was significant during initial chamber fill while CO_2

levels were rising, the second two minutes of exposure to increased CO_2, throughout the refill to normal room air, and while normal room air was maintained.

This pattern of results indicates that Lc/+ mice were impaired in responding to multiple aspects of this CO_2 challenge. Specifically, the significantly lowered response to increasing concentrations of CO_2 during the initial chamber fill indicates that Lc/+ mice were slower to initiate a compensatory increase in *f* in response to this challenge. That the genotypes did not differ significantly during the first two minutes of exposure to increased CO_2 shows that Lc/+ mice ultimately did initiate a compensatory response to the CO_2 challenge. However, Lc/+ mice then failed to maintain this compensatory increase during the second, two-minutes of exposure. This lack of maintenance in the compensatory response may indicate that Lc/+ mice were comparatively insensitive to rising CO_2 levels. As a function of this failure to maintain compensation it can be assumed that blood levels of CO_2 in Lc/+ mice became higher than those of control (Alheid & McCrimmon, 2009; Sherwood, 1997). Thus, the subsequent genotype related differences in *f* that occurred during chamber refill and maintenance with room air may additionally represent an inability to eliminate the increased blood levels of CO_2.

Hypoxia

When exposed to hypoxic conditions, both genotypes exhibited a gradual reduction in TV and *f* as O_2 concentration decreased. It should be noted that both the magnitude of changes in TV and *f* as well as the pattern of ventilatory decline observed in both genotypes can be considered to be relatively typical. Hypoxia, especially when O_2 levels do not decline below 10%, elicits a much less robust response than hypercapnia, as the respiratory and cardiovascular systems tend to be more receptive to changes in CO_2

than O_2 (Fong, 2010; Teppema & Dahan, 2010). Additionally, the hypoxic ventilatory decline seen across the O_2 challenges in both genotypes is likely related to their genetic background as it has previously been reported that this is a typical response in C57BL/6 mice (Chai, Gillombardo, Donovan, & Strohl; Tankersley et al., 2000).

Genotype-related differences were relatively small and infrequent during the hypoxic challenges. At 17 and 15% O_2, during, respectively, the return to room air (17% O_2) and while the chamber was refilling with 15% O_2, Lc/+ mice had significantly lower *f* than wildtype controls. Thus, it is possible that Lc/+ mice may have exhibited an impairment in the ability to recover from moderately reduced O_2 in comparison to WT mice.

Relation to Previous Research

It should be noted that the current results implicating cerebellar Purkinje cell loss in the differential reduction in responsiveness to hypercapnic conditions are significantly different from those previously reported in another animal model of Purkinje cell degeneration. In shaker mutant rats that exhibit varying levels of cerebellar Purkinje cell loss which begins seven weeks after birth, it has been reported that breath frequency during hypercapnia is augmented rather than reduced (Sarna & Hawkes; Xu, Zhou, & Frazier, 2004). Aside from species related differences, shaker mutant rats also differ from *Lurcher* mutants in both the temporal and spatial pattern of Purkinje cell loss. Shaker mutant rats comprise two groups, mild and strong, that differentially exhibit hereditary cerebellar Purkinje cell loss (Tolbert, Ewald, Gutting, & La Regina, 1995). The mild shaker group is characterized behaviorally by ataxic gait and exhibits cerebellar Purkinje cell loss that occurs randomly, primarily in the anterior lobe, and begins by PND 42 to 49

and culminates approximately by PND 90. The strong shaker is characterized behaviorally by both ataxic gait and severe body tremors, and additionally exhibits near-complete cerebellar Purkinje cell loss in the anterior lobe that occurs by PND 90. Accompanying the early anterior lobe loss, Purkinje cells in the posterior lobe (lobules VIIb, VIII, IXa-c) continue to degenerate throughout the life of this rat (Sarna & Hawkes, 2003; Tolbert et al., 1995).

In comparison, Lc/+ mice exhibit global loss of cerebellar Purkinje cells that begins almost immediately after birth as a result of spontaneous apoptosis, with nearly 100% Purkinje cell loss in the cerebellar cortex by PND 28 (Sarna & Hawkes, 2003; Zuo et al., 1997). The combination of differences in the topography and timing of Purkinje cell loss between Lc/+ mice and shaker mutant rats clearly indicates that interpretation of the role of developmental cerebellar damage on stressed breathing is complicated, and the relationship between cerebellar Purkinje cell loss and the response to hypercapnia is something other than a simple correlation between Purkinje cell number and respiratory response. Thus, the abovementioned difference in results highlights the need for further investigation of the effects of cerebellar Purkinje cell loss and challenged breathing.

There are a number of possible reasons why Lc/+ mice exhibit deficits in respiratory compensatory behaviors in response to hypercapnia, and to a lesser degree, hypoxia. It seems unlikely that these differences can be attributed to peripheral mechanisms. Diaphragm musculature in Lc/+ mice has been reported to be *more* fatigue resistant than that in WT mice which suggests that reduced muscle capability of the mutants dose not play a role (Hartmann, Martrette, & Westphal, 2001). Additionally, a motor deficit in Lc/+ mice appears unlikely as these animals were clearly capable of

achieving appropriate levels of TV and *f*. Thus, for example, TV in Lc/+ mice at 6% CO_2 clearly matched or exceeded TV in wildtype mice at 4% CO_2 (Figure B1, panel b & c).

A more likely explanation for the currently observed genotype-related differences involves one of the deep cerebellar nuclei, the fastigial nucleus. It has been previously reported that that this nucleus is involved in facilitating compensatory respiratory responses to hypercapnia, and to a lesser extent hypoxia (Hernandez, Xu, & Frazier, 2004; Xu et al., 2004). Cell body lesions of this nucleus had no effect on eupnoeic breathing but markedly reduced the respiratory response to increased concentrations of CO_2 (Martino et al., 2007). Purkinje cells output through all the deep cerebellar nuclei including the fastigial nucleus. In Lc/+ mice, as a function of loss of Purkinje cells and associated reductions in impulse traffic, the deep cerebellar nuclei exhibit a 60% reduction in volume (Heckroth, 1994). It is reasonable to assume that the shrinkage of the fastigial nucleus that occurs in Lc/+ mice likely also involves a loss of chemosensitivity that could result in an inability to respond appropriately to hypercapnic conditions.

Implications and Future Research

Disordered breathing is common across multiple disorders involving cerebellar pathology. For example Chiari Type II malformations and Joubert Syndrome are respectively associated with herniation or hypoplasia of the cerebellar vermis and both are characterized by sleep disordered breathing (Kijsirichareanchai, Limsuwat, Mankongpaisarnrung, Nantsupawat, & Nugent, 2013; Maria, Boltshauser, Palmer, & Tran). This association is noteworthy as the cerebellar vermis has been consistently recognized as an important area of interest in the respiratory response pattern to hypercapnia and air hunger (Evans et al., 2002; Parsons et al., 2001; Peiffer, Poline,

Thivard, Aubier, & Samson, 2001). Diffusion tensor imaging studies in persons diagnosed with congenital central hypoventilation syndrome (CCHS) have identified cerebellar abnormalities including myelin alterations of the cerebellar vermis and deep cerebellar nuclei (Kumar, Macey, Woo, Alger, & Harper, 2008). Additionally, persons with Fragile-X syndrome, the largest monogenetic cause of autism, exhibit focal cerebellar Purkinje cell loss and Bergmann gliosis and also show an increased incidence of sleep apnea as well as an increased likelihood of giving birth to children who die from SIDS (Fryns, Moerman, Gilis, d'Espallier, & Berghe, 1988; Sabaratnam, 2000).

Cerebellar neuropathology including cerebellar hypoplasia and reduced cerebellar Purkinje cell numbers are the most consistent neuropathologies linked to disorders within autism spectrum disorders (ASD; Bauman & Kemper, 1985; Bolduc et al., 2011; Courchesne, 1997; Courchesne et al., 1994; Eric Courchesne, Yeung-Courchesne, Hesselink, & Jernigan, 1988; DiCicco-Bloom et al., 2006; Limperopoulos et al., 2012; Palmen, van Engeland, Hof, & Schmitz, 2004; Whitney, Kemper, Rosene, Bauman, & Blatt, 2009). Although there have been no systematic comparisons of the incidence of breathing disorders in ASD it is important to note that sleep related disorders of stressed or challenged breathing, as well as SIDS have been reported in many clinical syndromes encompassed within the category of ASD. These disorders include phenylketonuria, Joubert syndrome, Fragile X, Angelman syndrome, Rett syndrome, and Mobius syndrome (Fryns, Moerman, Gilis, d'Espallier, & Berghe, 1988; Gilmore, Falace, Kanga, & Baumann, 1991; Ishikawa et al., 2008; Lioy, Wu, & Bissonnette, 2011; Miano et al., 2005; Schulte et al., 1973; Taddeucci, Bonuccelli, Mantellassi, Orsini, & Tarantino, 2010). Although neuropathologic studies of some of these disorders are rare, they appear

to involve varying types of cerebellar abnormalities that include extensive cerebellar neuron loss (Kornguth, Gilbert-Barness, Langer, & Hegstrand, 1992), agenesis or dysgenesis of the cerebellar vermis (Yachnis & Rorke, 1999), focal cerebellar Purkinje cell loss and Bergmann gliosis (Sabaratnam, 2000), cerebellar atrophy (Jay, Becker, Chan, & Perry, 1991), gliosis, hypoplasia and progressive atrophy in the cerebellum, including Purkinje cell loss (Murakami, Courchesne, Haas, Press, & Yeung-Courchesne, 1992; Oldfors et al., 1990), and cerebellar hypoplasia (Lengyel, Zaunbauer, Keller, & Gottlob, 1999; Nardelli, Vio, Ghersini, & Rizzuto, 1982). Although these disorders also show varying degrees of neuropathology in other brain areas, the common features linking all of these disorders are developmental cerebellar neuropathology, autistic symptomatology, and sleep disordered breathing.

The response to environmental changes in CO_2 and O_2 involve more than just respiratory compensation. Thus, both heart rate and blood pressure should also increase in response to rising levels of CO_2 (Alheid & McCrimmon, 2009; Sherwood, 1997). It would therefore be sensible to implement measures of heart rate and blood pressure compensation during respiratory challenges within animal models of cerebellar neuropathology involving Purkinje cells. It would also be interesting to examine blood oxygen saturation levels, however at this time no reliable method exists for measuring blood oxygen saturation in a mouse model while the animal is in an unrestrained WBP, and it has been shown that placing the animals in a restrained position for these measurements alters their natural response patterns (DeLorme & Moss, 2002). Additionally, to further investigate the relationship between developmental cerebellar neuropathology, sleep disordered breathing, ASD, and SIDS it would be important to

assess the cardiorespiratory compensatory responses to hypercapnic and hypoxic challenges in sleeping animals as well as in younger animals.

Conclusion

This experiment clearly indicates the need to further evaluate cerebellar pathology, more specifically Purkinje cell pathology, as a potential contributor to respiratory response and compensation during challenged breathing. This study served to examine whether cerebellar neuropathology in the form of global developmental Purkinje cell loss would negatively influence respiratory response to and recovery from acute hypercapnic and hypoxic challenges. Overall, Lc/+ mice consistently displayed a reduced response to and recovery from hypercapnic and, to a lesser degree hypoxic, challenges as compared to littermate WT controls.

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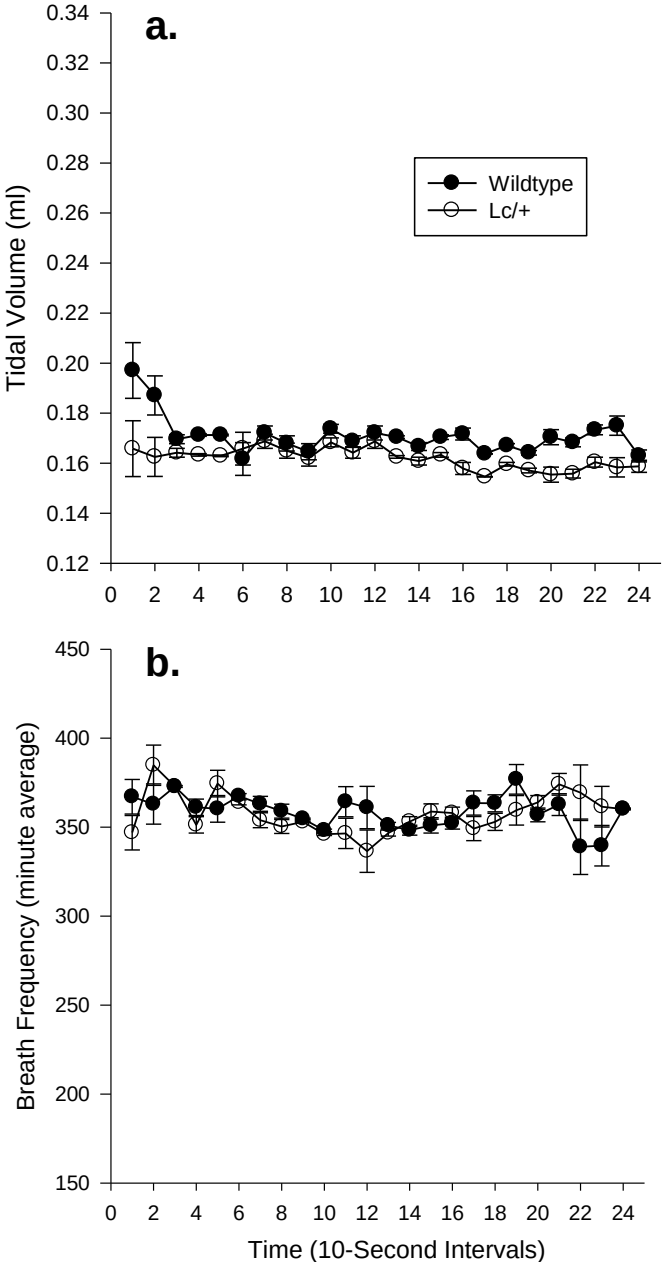

Figure A1. Baseline data over 24, 10 second intervals at normal room air (21% O_2, 0% CO_2, Balance N_2) in *Lurcher* mutant (Lc/+) and wildtype control mice. The vertical lines indicate the SEM.

Appendix B

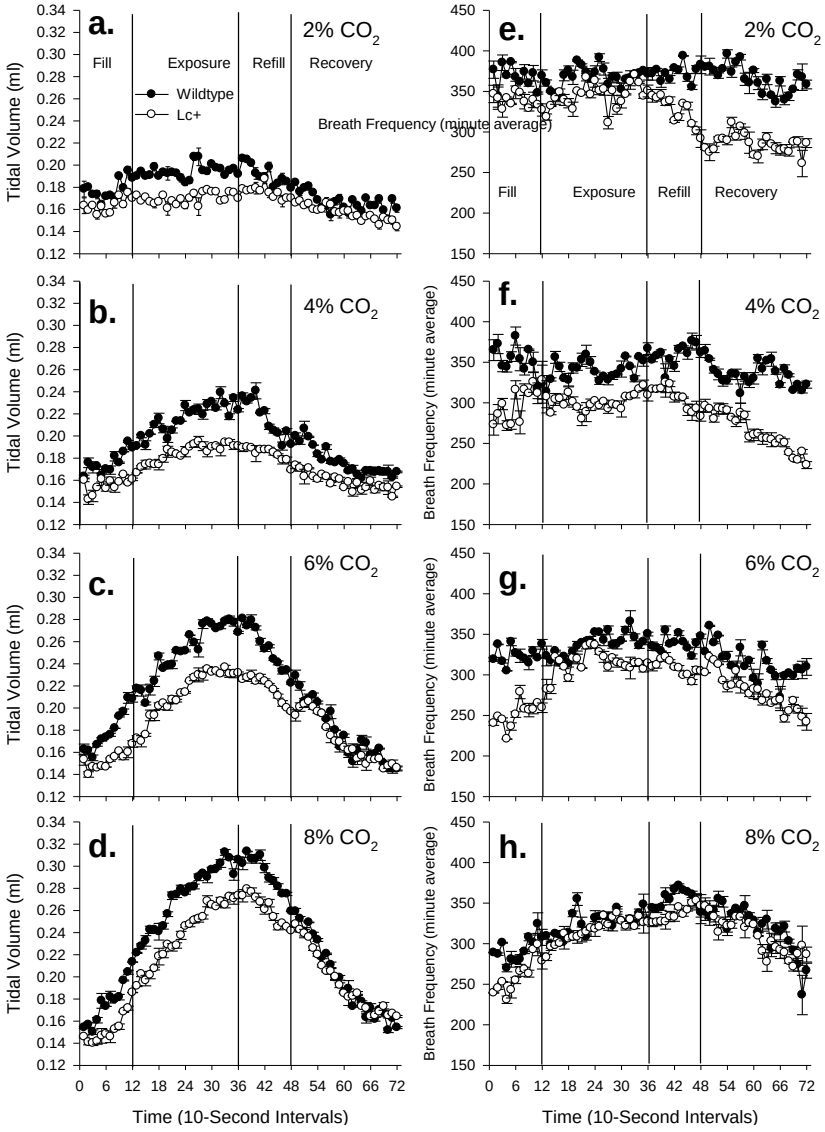

Figure B1. Tidal volume (Left column) and breath frequency (Right column) in response to increasing concentrations of CO_2 (percentages indicated in the upper right corner of each panel figure). The horizontal axis in each figure depicts time intervals across 4 time blocks that correspond to chamber **Fill** with CO_2, **Exposure** to increased levels of CO_2, chamber **Refill** with room air and **Recovery** with room air maintained. Exposure and Recovery conditions were additionally subdivided into two, two-minute blocks (e.g. Exposure 1 & 2) for the purposes of statistical analyses. See text for further details. The vertical lines indicate the SEM.

Appendix C

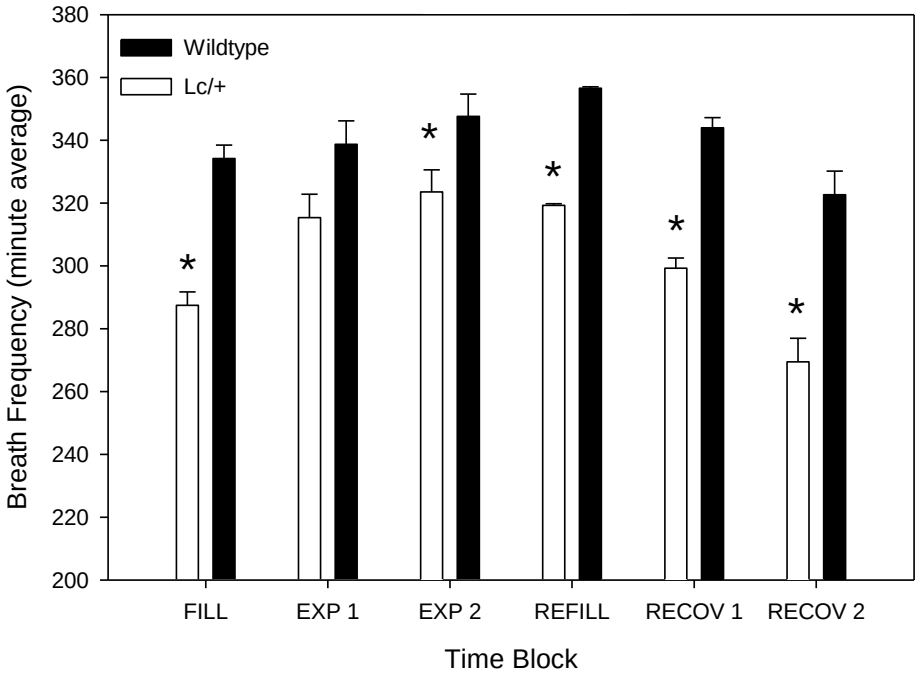

Figure C1. Genotypic differences in breath frequency among the six time blocks collapsed across all CO_2 challenges. The vertical lines indicate the SEM.

Appendix D

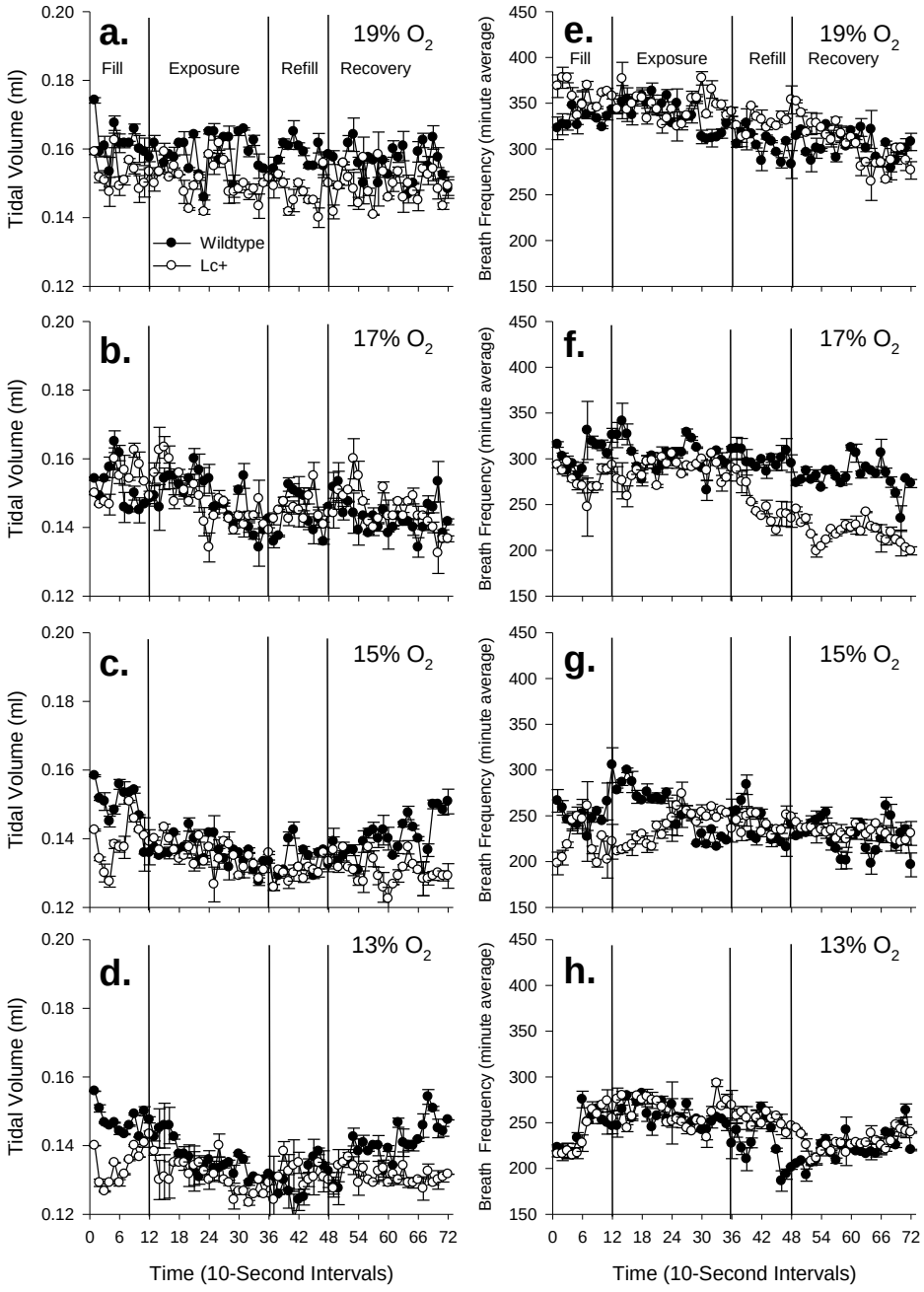

Figure D1. Tidal volume (Left column) and breath frequency (Right column) in response to decreasing concentrations of O_2 (percentages indicated in the upper right corner of each panel figure). The horizontal axis in each figure depicts time intervals across 4 time blocks that correspond to chamber **Fill** with reduced concentration of O_2, **Exposure** to reduced levels of O_2, chamber **Refill** with room air and **Recovery** with room air maintained. Exposure and Recovery conditions were additionally subdivided into two, two-minute blocks (e.g. Exposure 1 & 2) for the purposes of statistical analyses. See text for further details. The vertical lines indicate the SEM.

THE UNIVERSITY OF MEMPHIS

IACUC PROTOCOL ACTION FORM

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

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

0721 (Mouse model of SIDS …)

☒ Your proposal is approved for the following period:

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

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

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

From: To:

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

From: To:

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

Prof. Guy Mittleman, Chair of the IACUC

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