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Conditional Loss of Engrailed 1/2 in Rhombic Lip-Derived Neurons Increases Intrinsic Rhythmicity and Decreases Overall Variability of Eupneic Respiration

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Abstract

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UNIVERSITY OF TENNESSEE HEALTH SCIENCE CENTER

MASTER OF SCIENCE THESIS

**Conditional Loss of *Engrailed 1/2* in Rhombic
Lip-Derived Neurons Increases Intrinsic Rhythmicity
and Decreases Overall Variability of Eupneic
Respiration**

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This work, as in all my life, is dedicated to my Creator. "And whatever you do, in word or deed, do everything in the name of the Lord Jesus, giving thanks to God the Father through him." Colossians 3:17 ESV

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Evidence for a cerebellar role during cardiopulmonary challenges has long been established, but investigation into cerebellar involvement in eupneic breathing has been inconclusive. Given the view of the cerebellum (CRB) as a temporally coordinating structure, any investigation into the CRB during respiration must evaluate rhythm and variability of the respiratory sequence. In this study, we chose an elegant model of cerebellar neuropathology, *Atoh1-En1/2* CKO, where mutant animals have conditional loss of the developmental patterning gene *Engrailed 1/2* in rhombic lip-lineage neurons and exhibit a proportional scaling-down of neuron number in hypoplastic lobules of the CRB. We utilized whole-body unrestrained plethysmography to measure respiration during eupnea, and evaluated the respiratory sequence of mutant animals and their control littermates using the average rate, the coefficient of variation (CV), and a unique measure of intrinsic rhythmicity called CV2. Linear regression analyses revealed that mutant animals had decreased overall variability and increased intrinsic rhythmicity (as measured by CV and CV2, respectively) compared to their control littermates, but we found no effect of strain on average respiratory rate. Analysis also revealed modestly decreased respiratory rates, increased CV, and increased CV2 in female animals, independent of strain. These results align well with previously reported studies and add new insight into CRB involvement in eupneic respiratory rhythmicity. Although not investigated in this study, future works should consider the coordination of breathing, licking, swallowing, and whisking in order to build a more complete understanding of the relationship between the CRB and respiration.

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List of Abbreviations

| | |
|--------------|--------------------------------------|
| <i>Atoh1</i> | <i>Atonal homolog 1</i> |
| CKO | Conditional knockout |
| CRB | Cerebellum |
| CV | Coefficient of variation |
| DCN | Deep cerebellar nuclei |
| eCN | Excitatory cerebellar nuclei neurons |
| <i>En1/2</i> | <i>Engrailed 1/2</i> |
| IRI | Inter-respiratory interval |
| mDCN | Medial deep cerebellar nucleus |
| PC | Purkinje cell |

Chapter 1

Introduction

The involvement of the cerebellum (CRB) in cardiopulmonary function such as blood pressure regulation and respiration has long been established and is evidenced by a wealth of anatomical and neuropathological data [2, 40, 23, 16, 57, 19, 9, 31, 13, 43]. While many have supported CRB involvement in breathing during respiratory challenges, like hunger for air and hypoxia, a similar relationship has not been found when evaluating normal breathing, also called eupneic breathing or eupnea [35, 61, 39, 31, 8]. This study seeks to investigate the cerebellar role during eupneic respiration using a mouse model of abnormal cerebellar development. This model, with conditional loss of the developmental patterning genes *Engrailed 1* and 2 (*En1/2*) in rhombic lip-derived neurons, presents a great opportunity to further our understanding of this cerebellar role. Mutant animals have demonstrated motor deficits, a proportional scaling down of neuron number in hypoplastic lobules of the CRB, and significantly reduced neuron number in the medial and intermediate deep cerebellar nuclei (DCN) [53, 38], but no studies to date have evaluated the effect of the conditional loss of *En1/2* on eupneic breathing in these animals.

Many studies which have failed to implicate the CRB during eupneic respiration have not evaluated changes to the respiratory pattern. Therefore, our lab recently investigated changes to respiratory patterns during eupneic breathing using a mouse model with Purkinje cell (PC) silencing [28]. Unlike other common models of ataxia with broad neuropathology, this mouse model of cerebellar ataxia lacks synaptic vesicle transmission only in PCs, thereby preventing output from the cerebellar cortex [51]. The lack of PC output did not affect the average respiratory rate, as measured by the mean inter-respiratory interval (IRI), nor did it affect the coefficient of variation (CV). While CV is an effective measure of overall variability, it is not sensitive to either brief variability in adjacent respiratory intervals or slow rate changes. CV is calculated from the total IRI distribution, but does not take into account the chronological order of IRIs. To investigate whether this intrinsic rhythmicity was affected, we utilized a measure called CV2, which was first described by Holt *et al.* in 1996 to analyze intrinsic variability in visual cortex neuron spiking in cats [22]. CV2 is calculated by finding the standard deviation of adjacent IRIs, dividing by their mean, and scaling by $\sqrt{2}$ (Eq. 2.1) [22]. The CV2 of a sequence, then, is the mean of all CV2s calculated for each adjacent pair of IRIs in the sequence. We found that the absence of cerebellar cortical output led to significantly decreased CV2 in mutants as

compared to control animals, which indicates increased intrinsic respiratory rhythmicity [28]. These novel results confirmed the importance of the cerebellum in coordinating respiratory rhythms during eupneic breathing.

Whereas our previous study emphasized the importance of the cerebellar cortex during eupnea, this study aimed to investigate the role of excitatory cerebellar nuclei (eCN) neurons. The eCN are not only the final output of the cerebellum, but they integrate information from PC as well as climbing fiber and mossy fiber collaterals [41, 45, 26]. Lu *et al.* has previously shown that there are robust projections from medial DCN (mDCN) and some projections from intermediate DCN to the ventromedial reticular formation, a structure involved in respiratory and orofacial motor control [30, 44, 7, 12, 63]. In addition, they found that a subset of mDCN neurons showed firing patterns which were significantly correlated with respiration [30]. Therefore, this present study used a unique mouse model developed by Orvis *et al.* [38] with conditional loss of function of the developmental patterning genes *Engrailed 1* and 2 (*En1/2*) in excitatory cerebellar neurons. This model (*Atoh1-En1/2* CKO) is especially appropriate in that normal cerebellar cytoarchitecture is preserved, with only proportional scaling down of neuron number in hypoplastic lobules and a reduction of the intermediate and medial DCN [53]. This model has shown deficits in motor behaviors [53], but no studies to date have examined the effect of conditional loss of *En1/2* in excitatory cerebellar neurons on respiratory rhythmicity. Given the results from our recent study and our understanding of the *Atoh1-En1/2* CKO mouse model, we hypothesized that conditional loss of *En 1/2* in excitatory cerebellar neurons would result in increased intrinsic rhythmicity (CV2), but no change in overall variability (CV) or respiratory rate.

1.1 Evidence for Cerebellar Involvement in Respiratory Function

Regions associated with respiratory pattern generation are primarily located in the brainstem, including the pre-Bötzinger complex, Bötzinger complex, retrotrapezoid nucleus, and the rostral, caudal and pontine respiratory groups [13]. Although most early research involving the cerebellum focused on its role in voluntary functions [34], scientists have also investigated a putative role in autonomic functions such as respiration [35]. The mechanism of involvement is still being elucidated, but there is growing electrophysiological, anatomical, and functional evidence to support a cerebellar role in breathing. Specifically pointing to the mDCN. In a 1992 study using alert cats, Gruart and Delgado-Garcia recorded neurons in the mDCN which were temporally aligned with eupneic respiration [17]. In addition, several studies found that stimulating the mDCN modulated phrenic nerve activity and respiratory rate [54, 58, 59]. Lu *et al.* further supported mDCN involvement, recording mDCN single unit activity which was correlated with respiration [30]. Using retrograde and anterograde tract tracing techniques, their study also found projections from the mDCN and intermediate DCN to brainstem regions containing respiratory pattern generators, specifically the ventromedial medullary reticular formation.

In addition, the cerebellum has been implicated in several studies investigating cardiopulmonary function. Harper *et al.* [19] used functional magnetic resonance imaging to identify structures that become differentially activated (as compared to baseline) during cardiopulmonary challenges. Specifically, they found that inspiratory loading and hypercapnic stimulation resulted in recruitment of the deep cerebellar nuclei and ventral cerebellum, respectively. Others have reported cerebellar activation during procedures which increased blood pressure, hypercapnia, and expiratory loading [2, 23, 16, 57]. While there is a clear involvement of the cerebellum during cardiopulmonary challenges, these studies have failed to implicate the cerebellum during eupnea [35, 61, 39, 60, 31, 8]. One study by Xu and Frazier investigated respiratory variables such as tidal volume in anesthetized cats, comparing normal animals to cats either post-cerebellectomy or with thermal lesions of the mDCN [57]. Progressive and transient hypoxia revealed changes in respiratory variables, but no difference between animals was found during eupneic breathing. This trend has continued even in recent years, as in a 2016 investigation of breathing in a mouse model of cerebellar ataxia called *Lurcher*, which has total Purkinje cell loss [8]. Again, differences between control and mutant animals were only significant during respiratory challenges.

These studies were valuable in furthering our understanding of cerebellar function as it relates to cardiopulmonary challenges, but they form an incomplete picture of the cerebellar role in eupneic respiration because respiratory sequence variability was not considered. The cerebellum can be viewed as a highly precise temporally coordinating structure, which will be described in greater detail below. Two separate studies, using the same mouse model, discovered that animals without PC transmission have altered eupneic respiratory patterns compared to control littermates [20, 28]. In van der Heijden and Zoghbi, PC silencing resulted in increased inter-breath irregularity during normoxic conditions. Therefore, to fully understand the cerebellar role during eupneic respiration, the finer aspects of respiratory timing must also be evaluated.

1.2 The Cerebellum as Timekeeper and the Cerebellar Microcircuit

As our understanding of cerebellar anatomy and physiology has developed, experts have grown to view the cerebellum as a timekeeper, precisely coordinating other structures to facilitate not only fine motor control but also complex cognitive functions [1]. In a recent consensus paper on the subject, Bareš *et al.* argue that the high density of neurons, strictly organized microcircuitry, and vast interconnectivity with other cortical structures make the cerebellum uniquely suited to this role. Within the scope of this study, the cerebellar microcircuit is of particular importance.

Sensory and postural information from the cortex, spinal cord and vestibular system are routed through the pons and enter the cerebellum as mossy fibers [41]. These mossy fibers synapse primarily with granule cells in the internal granule cell layer, and

granule cells extend their axons radially to the molecular layer and then bifurcate as parallel fibers [41]. The parallel fibers travel along the mediolateral axis, synapsing with PC dendritic arbors — a single rat PC can receive synaptic connections from about 175,000 parallel fibers [41, 36]. In contrast, climbing fibers form strong connections with 5-10 PCs, with one PC only receiving input from a single climbing fiber [48]. These climbing fibers carry sensorimotor and cognitive information from the inferior olive, and they are responsible for the distinct complex spike that be seen in PC electrophysiological recordings [48]. PCs then form the sole output of the cerebellar cortex and project to the deep cerebellar nuclei, which are made up of inhibitory neurons targeting the inferior olive and the eCN [15, 49]. The eCN integrate information from not only the PCs but from mossy fiber and climbing fiber collaterals, then exit the cerebellum as its final output to the thalamus and other brainstem areas [41, 45, 26].

It is agreed that the cerebellar microcircuit is essential for cerebellar temporal coordination, but how this role affects eupneic respiration is not well understood. One important study investigated a putative role of the mDCN in coordinating eupneic respiration with orofacial movements, such as licking and swallowing [30]. In addition to the previously described anatomical evidence, they found a subset of mDCN neurons with spiking patterns which were significantly correlated with both respiration and swallowing. Furthermore, our research in Liu *et al.* highlighted the significance of PC output in the intrinsic rhythmicity of breathing [28]. This study then seeks to further our understanding by investigating the eCN and the effects of their abnormal development on the respiratory rhythm.

1.3 Development and Characteristics of the *Atoh1-En1/2* CKO Strain

As the final output of the cerebellum, the excitatory cerebellar nuclei (eCN) must be investigated to fully understand the cerebellar role in respiration. To do so, we will utilize a unique mouse model originated by Orvis *et al.* with conditional loss of the developmental patterning genes *Engrailed 1/2* (*En1/2*) in rhombic lip-derived neurons [38]. These animals were developed using a *Cre/lox* system which conditionally knocks out *En1/2* in excitatory cerebellar neurons, including granule cells and the eCN, using the *Atoh1* promoter (*Atoh1-En1/2* CKO).

Engrailed 1 and *2* are homeobox transcription factors which have been shown to be essential for normal development of the brain [55, 25, 53]. They have overlapping but not identical expression patterns, primarily in the developing midbrain and hindbrain [11, 55]. Mutant mouse strains have revealed that *En1/2* are crucial in the CRB for normal molecular patterning of the PC parasagittal stripes, foliation, and afferent circuit maps [47, 10, 46].

Given the widespread role of *En1/2* in the developing brain, we have selected a mouse model which conditionally knocks out *En1/2* expression in rhombic lip-derived neurons. The cerebellar rhombic lip and ventricular zone are the complementary progenitor zones which develop into the cerebellum [26]. The ventricular zone progenitors

give rise to cerebellar GABAergic neurons, including Purkinje cells, inhibitory interneurons, and Bergmann glia [26]. Rhombic lip progenitor cells express the transcription factor *Atonal homolog 1 (Atoh1)* and give rise to the glutamatergic neurons of the DCN, granule cells, as well as several brainstem nuclei [32, 50]. Fate mapping studies helped clearly define structures originating from the rhombic lip, and a good summary of these results can be found in Wingate 2005 [56]. Due to the importance of brainstem structures in governing respiration, it is unsurprising that *Atoh1*-null mice do not survive postnatally due to respiratory failure [24].

In terms of cerebellar size and foliation, the *Atoh1-En1/2* CKO strain suffers proportional scaling down of neuron number in hypoplastic lobules of the anterior and central vermis and paravermis, as well as several shortened or missing fissures [38, 53]. In addition, the medial and intermediate deep cerebellar nuclei are greatly reduced in size, yet cerebellar cytoarchitecture is maintained [53]. This crucial aspect of this model sets it apart from other cerebellar mutant strains and makes it ideal for this investigation. Thus the *Atoh1-En1/2* CKO strain, with its unique developmental irregularities and preserved cytoarchitecture, lends great potential to this investigation of a cerebellar role in eupneic respiration.

Chapter 2

Materials and Methods

2.1 Animals

All animals were bred and genotyped in the lab of Dr. Alexandra Joyner (Memorial Sloan Kettering Cancer Center, USA) and then transferred to the animal care facilities at the University of Tennessee Health Science Center (UTHSC). Use of animals and all experimental procedures were performed under the approval of the Institutional Animal Care and Use Committee of UTHSC. In total, 39 animals were used for analysis (details in **Table 2.1**).

Table 2.1: Mouse group composition and means.

| Variables | All (N = 39) | Mutant (N = 19) | Control (N = 20) |
|---------------------------------|---------------|-----------------|------------------|
| Sex^a | | | |
| Female | 24 (61.54%) | 12 (30.77%) | 12 (30.77%) |
| Male | 15 (38.46%) | 7 (17.95%) | 8 (20.51%) |
| Mean IRI (s)^b | 0.164 (0.035) | 0.156 (0.030) | 0.172 (0.039) |
| Rate | 6.30 (1.12) | 6.58 (1.06) | 6.03 (1.13) |
| CV | 0.37 (0.06) | 0.34 (0.07) | 0.39 (0.06) |
| CV2 | 0.23 (0.04) | 0.21 (0.03) | 0.25 (0.03) |

^a Count and (%) for discrete variables

^b Mean and (standard deviation) for continuous variables

Originally developed and described by Orvis *et al.* [38], this strain uses a *Cre/lox* system and the *Atoh1* promoter to conditionally knock out the *Engrailed 1/2* genes in rhombic lip-derived excitatory cerebellar neurons. Mutant animals (*Atoh1-Cre/+; En1 fx/fx; En2 fx/fx*) were compared to their control littermates (*En1 fx/fx; En2 fx/fx*). We recorded animals at 4-5 months old, corresponding to mature adulthood in mice [14].

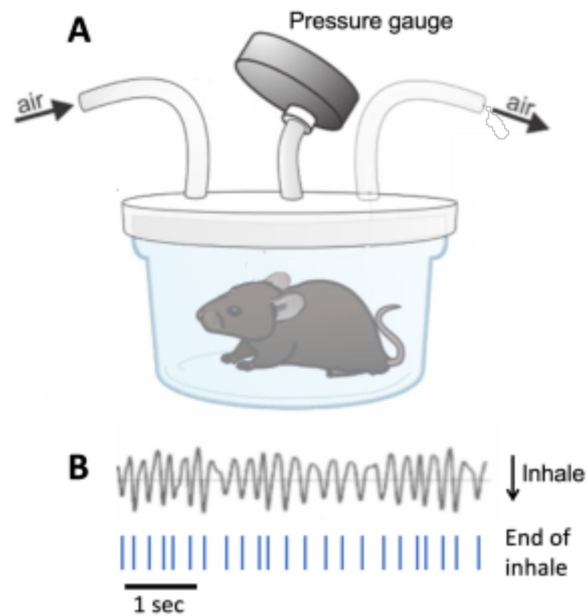


Figure 2.1: Whole-body unrestrained plethysmograph recording. A) Example plethysmograph chamber. Pressure changes due to respiration are reflected by voltage changes, shown in B. B) In this illustration, voltage decreases as pressure increases due to respiration (the reverse is true in the present study - voltage increased with pressure increase.) These pressure increases are then marked, representing the end of inspiration. The sequence of marks is then used for all further analysis. Reprinted with open access permission. Liu *et al.* [28]

2.2 Plethysmograph Recording

To record respiratory patterns under eupneic conditions, the protocol described previously was followed [28]. In short, we used a plethysmograph chamber as shown in **Figure 2.1A**, constructed using an airtight container with constant air flow and a pressure transducer attached. Animals were recorded individually, an opaque covering was placed over the entire apparatus, and the animal's breathing was recorded for 30 minutes using Spike2 recording software (Cambridge Electronic Design, UK). As the animal inhales, pressure inside the chamber increases slightly, causing the voltage to increase (note: **Figure 2.1B** shows voltage decreasing with inspiration). The peaks, or end of inspiration, were marked and this sequence was used for all further analysis. This included both normal breathing and sniffing behavior under normoxic conditions. Between recording each animal, the chamber was cleaned with a 70% ethanol solution and allowed to dry.

2.3 Analysis

In Spike2, the raw signal was first processed using a FIR Butterworth low pass filter with a cutoff at 20 Hz to remove noise without distorting the respiratory data, as suggested by Lim *et al.* [27]. To remove very low frequency (< 1 Hz) drift, the built-in Spike2 DC remove channel process was utilized (time constant = 0.1 seconds). Signal peaks were marked using a minimum interval of 0.06 seconds to exclude local maxima which do not correspond to the end of inspiration, given that murine respiratory rates vary from 1-12 Hz (including sniffing behavior) [52, 51]. Minimum peak height varied based on the amplitude of individual recordings, but ranged from 0.15-0.35 Volts. Inter-respiratory intervals (IRI) were determined by calculating the time between subsequent end-of-inspiration marks (Δt). CV was calculated as the standard deviation of the IRI distribution divided by the mean. Finally CV2 [22], which compares subsequent IRIs, was calculated using Eq. 2.1.

$$CV2 = \frac{2|\Delta t_{(i+1)} - \Delta t_{(i)}|}{\Delta t_{(i+1)} + \Delta t_{(i)}} \quad (\text{Eq. 2.1})$$

To preserve data integrity, it was necessary to exclude from analysis periods of noise, which can be caused by the animal eliminating or by blocking the input or output tubes with its body [27]. For each mouse, the CV, CV2, and mean IRI of five respiratory sequences (each sixty seconds long) were calculated independently and then averaged. All calculations and statistical analyses were performed using MATLAB scripts (MathWorks, USA).

2.4 Statistics

First, the response variables were tested to ensure that they were appropriate for linear regression. In the following tests for normality and homoscedasticity, data for each response variable were treated as a single group (for example, all CV data was grouped regardless of sex or strain). First, response variables were evaluated to ensure normality using quantile-quantile (QQ) plots, which compare the distribution of a data set with the normal distribution. The Breusch-Pagan test was used to evaluate the data for homoscedasticity, where the null hypothesis is that data are homoscedastic and a p-value ≥ 0.05 indicates heteroscedasticity [3, 33]. To determine the effect of sex and strain on response variables (CV, CV2, and mean IRI), linear regression analysis was applied. Models were fitted to relate response variables to predictor variables (sex and strain). Insignificant predictor variables ($p \geq 0.05$) were removed to increase degrees of freedom and improve the model. Model fit was confirmed using residual plots.

Chapter 3

Results

3.1 Conditional Loss of *En1/2* in Excitatory Cerebellar Neurons Has No Effect on Respiratory Rate

To evaluate whether conditional loss of *En1/2* in eCN has an effect on average respiratory rate during eupnea, we intended to use mean IRI as in Liu *et al.* 2020 [28]. However, the QQ plot of IRI data, as well as the total IRI distribution (including all IRIs instead of averages) both showed clear right skews, shown in **Figure 3.1**, indicating that the data was not normally distributed.

This could be due in part to the artificial IRI minimum of 0.06 s, which was enforced to exclude intervals which are physiologically impossible. In addition, the Breusch-Pagan test revealed that IRI data were not homoscedastic ($p = 0.045$). Because of this right skew and failure to meet standards of homoscedasticity, the IRI data could not satisfy the assumptions of linear regression. To improve the model, a nonlinear transformation was applied by using the reciprocal of the mean IRI, which in this case is the physiologically relevant measure of rate. Again, a QQ plot and the Breusch-Pagan test were employed, revealing that rate was homoscedastic ($p = 0.056$) and better approximated a normal distribution as seen in **Figure 3.2B**.

Therefore, we determined that rate was a better candidate for linear regression and used rate as the response variable rather than mean IRI. This model revealed no significant effect of strain on average rate ($p = 0.088$, adjusted for sex), so strain was removed from the model to improve fit. Female animals showed significantly slower respiratory rates ($p = 0.019$) than males, independent of strain. This result was opposite to what we predicted in terms of sex, based on Liu *et al.* 2020 [28]. Raw residuals were plotted against fitted values, shown in **Figure 3.2C**, which revealed that female animals had greater residual variability than males. This can also be seen in the spread of rate values as shown in the boxplot in **Figure 3.2A**. Regardless, the effect of sex was modest ($\beta = -0.817$) and average rates were 6.81 Hz for females and 5.99 Hz for males. Our hypothesis was supported, however, in that conditional loss of *En1/2* in the eCN did not have a significant effect on average respiratory rate. To be sure that the sex difference in rate did not influence CV or CV2, rate was initially included as a predictor variable in the remaining linear regressions.

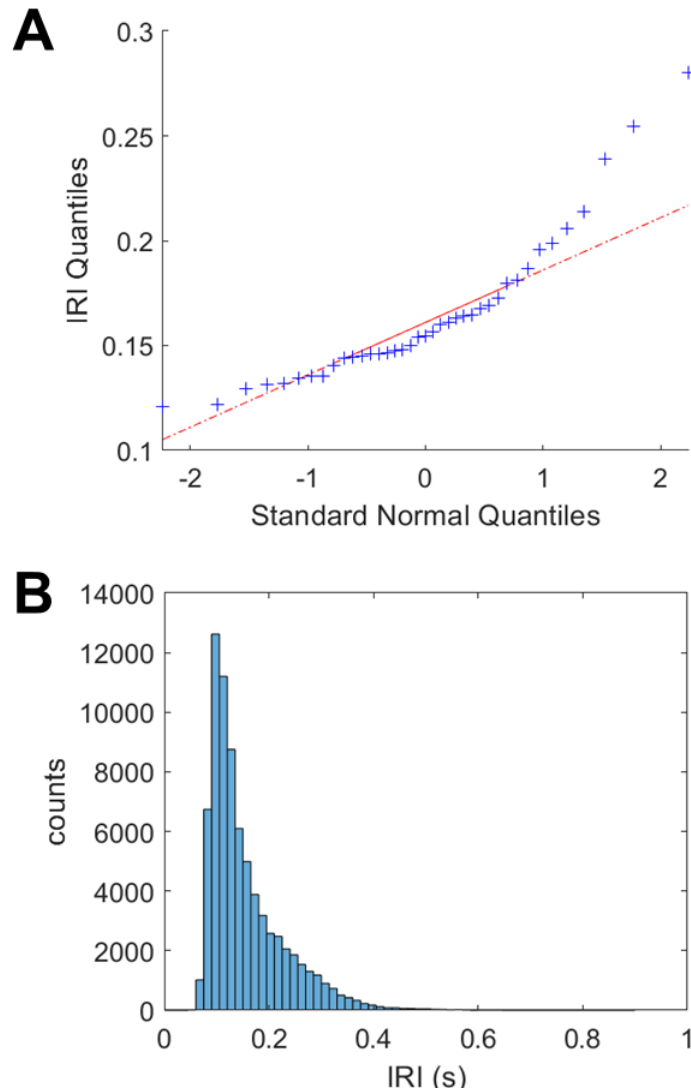


Figure 3.1: IRI data do not fit assumptions of linear regression.

A) Quantile-quantile plot of average IRI data versus standard normal distribution reveals deviation from normality. Blue plus signs indicate quantiles, dotted red line represents the plot of the line if data has a perfectly normal distribution. B) Histogram of total IRI data (not averages) with a bin size of 0.015 seconds. The right skew and artificial cutoff at 0.06 seconds can be clearly seen.

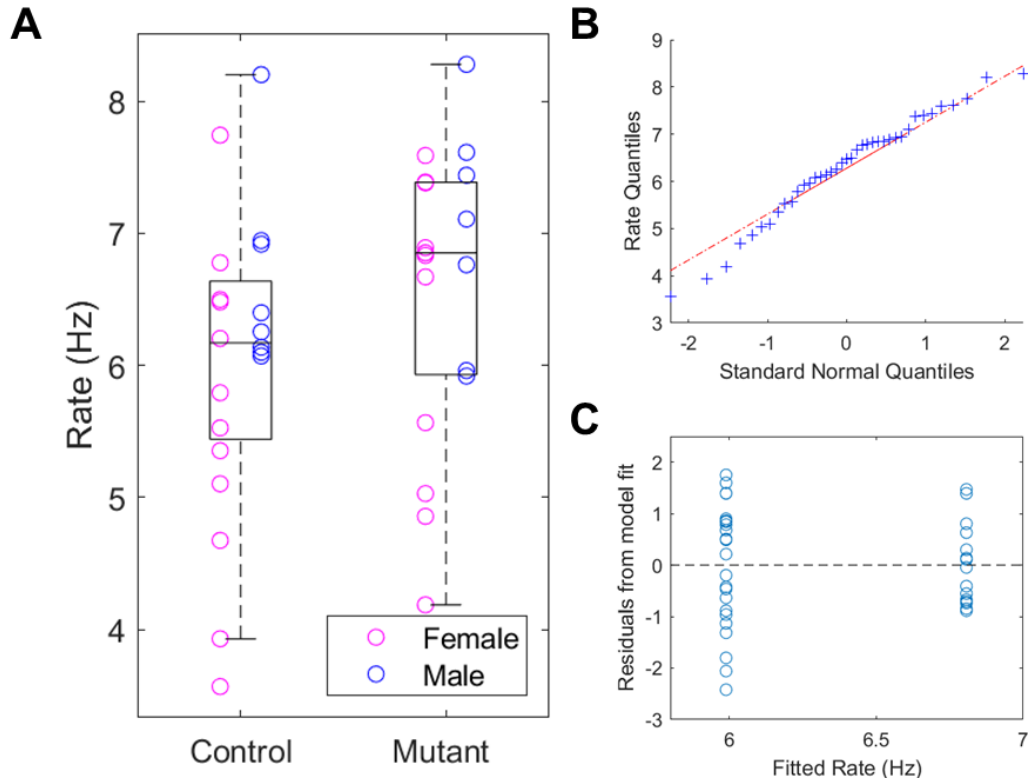


Figure 3.2: Average respiratory rate is influenced by sex and not strain.

A) Boxplot showing the average respiratory rates of individual mice (open circles). There was no significant effect of strain on respiratory rate ($p = 0.088$, adjusted for sex). After strain was removed from model, sex remained a significant predictor of average rate ($p = 0.019$). The upper, middle, and lower box limits indicate the 75th percentile, median, and 25th percentile, respectively. The whiskers represent $\pm 2.7\sigma$, encompassing 99.3% of the data if the data are normally distributed. B) Quantile-quantile plot of rate data versus standard normal distribution. Blue plus signs indicate quantiles, dotted red line represents the plot of the line if data has a perfectly normal distribution. C) Plot of residuals versus fitted rate, based on the linear model with sex as the only predictor of rate. Ideally, residuals are distributed equally for both males and females with no clear trend. Actual data shows an apparent increased spread of residuals for female data as compared to males. Fitted rate values: all female, 5.99 Hz; all male, 6.81 Hz.

Rate was not significant in either case ($p > 0.05$, data not shown) so it was removed from the models.

3.2 Mice with Conditional Loss of *En1/2* in Excitatory Cerebellar Neurons Show Decreased Overall Respiratory Variability

Next, we investigated if the conditional loss of *En1/2* in eCN affects eupneic respiratory variability using the coefficient of variation of IRIs. The QQ plot revealed that CV values were basically normally distributed (**Figure 3.3B**) and Breusch-Pagan test confirmed homoscedasticity ($p = 0.66$).

The residual plot in **Figure 3.3C** further confirmed appropriate model fit as there was no apparent trend to the residuals. There was a significant effect of both sex and strain on CV ($p = 0.0036$ and $p = 0.021$, respectively), with mutants animals having decreased CV as compared to control littermates, and males having decreased CV compared to females as can be seen in **Figure 3.3A**. In contrast to our expectations, this indicated that conditional loss of *En1/2* in eCN significantly decreased overall respiratory variability during eupnea, and indicates decreased variability in males compared to females.

3.3 Mice with Conditional Loss of *En1/2* in Excitatory Cerebellar Neurons Show Increased Intrinsic Respiratory Rhythmicity

Finally, we sought to evaluate the effect of conditional loss of *En1/2* in the eCN on intrinsic respiratory rhythmicity during eupnea. As with rate and CV, CV2 data was also confirmed to be normal (QQ plot in **Figure 3.4B**) and homoscedastic ($p = 0.29$, Breusch-Pagan test), and there was no pattern to the residual plot (**Figure 3.4C**).

Similar to CV, linear regression of CV2 revealed a significant effect of both sex and strain (**Figure 3.4A**). Mutant animals had significantly decreased CV2 values compared to control littermates ($p \ll 0.001$) and males had significantly decreased CV2 values compared to females ($p = 0.0025$). In other words, conditional loss of *En1/2* in the eCN resulted in increased intrinsic respiratory rhythmicity during eupnea.

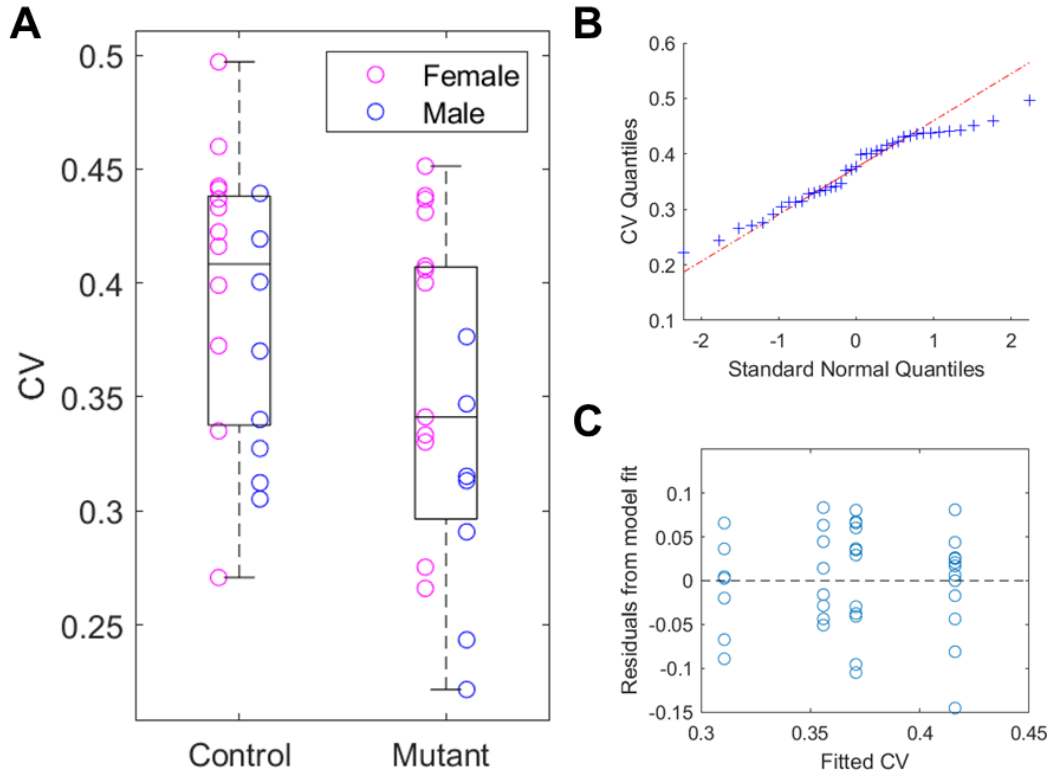


Figure 3.3: CV of IRI distribution is influenced by both sex and strain. A) Boxplot showing the CV of the IRI distribution of individual mice (open circles). Linear regression revealed a significant effect of both sex ($p = 0.0036$) and strain ($p = 0.021$) on CV. The upper, middle, and lower box limits indicate the 75th percentile, median, and 25th percentile, respectively. The whiskers represent $\pm 2.7\sigma$, encompassing 99.3% of the data if the data are normally distributed. B) Quantile-quantile plot of CV data versus standard normal distribution. Blue plus signs indicate quantiles, dotted red line represents the plot of the line if data has a perfectly normal distribution. C) Plot of residuals versus fitted CV. The four groups represented in fitted values are due to the fact that there can only be four possible combinations of the predictors sex and strain. Ideally, residuals are distributed equally amongst groups with no clear trend. Actual data shows no obvious trend, indicating good model fit. Fitted CV values: male mutant, 0.311; male control, 0.356; female mutant, 0.371; female control, 0.416.

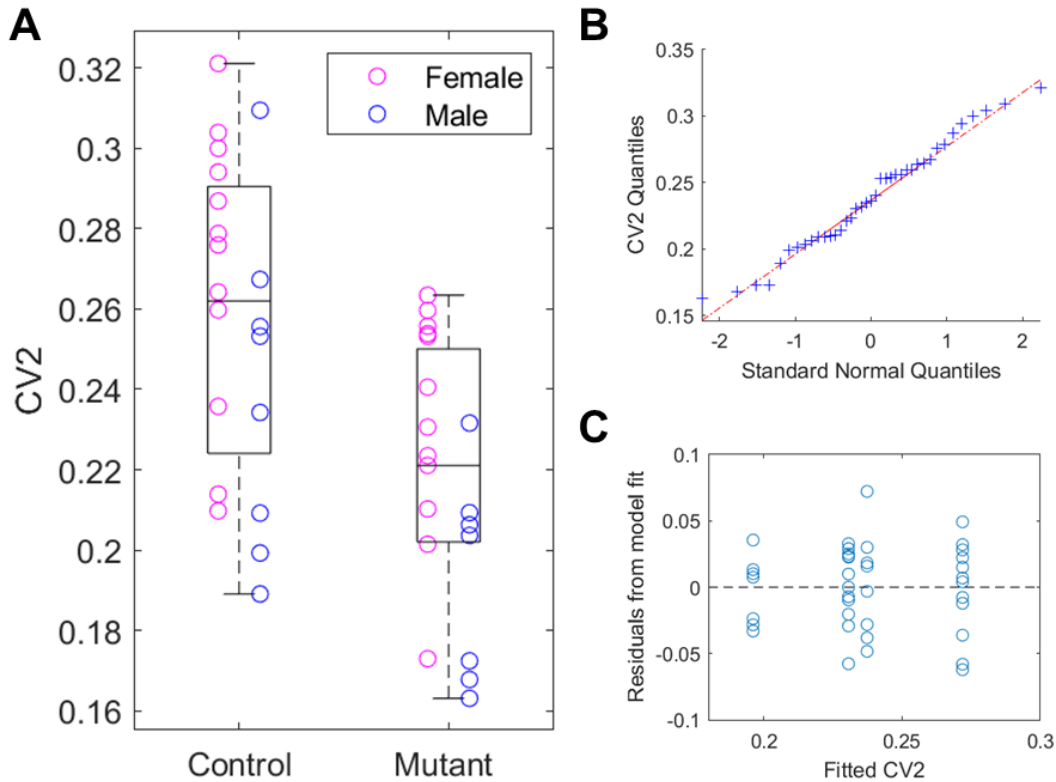


Figure 3.4: Mean CV2 is influenced by both sex and strain. A) Boxplot showing the mean CV2 of individual mice (open circles). Linear regression revealed a significant effect of both sex ($p = 0.0025$) and strain ($p < 0.001$) on CV2. The upper, middle, and lower box limits indicate the 75th percentile, median, and 25th percentile, respectively. The whiskers represent $\pm 2.7\sigma$, encompassing 99.3% of the data if the data are normally distributed. B) Quantile-quantile plot of CV2 data versus standard normal distribution. Blue plus signs indicate quantiles, dotted red line represents the plot of the line if data has a perfectly normal distribution. C) Plot of residuals versus fitted CV2. The four groups represented in fitted values are due to the fact that there can only be four possible combinations of the predictors, sex and strain. Ideally, residuals are distributed equally amongst groups with no clear trend. Actual data shows no obvious trend, indicating good model fit. Fitted CV2 values: male mutant, 0.196; male control, 0.237; female mutant, 0.231; female control, 0.272.

Chapter 4

Discussion

Based on our previous studies which demonstrated the importance of the cerebellar cortex during eupneic breathing [28] and established evidence for a relationship between the medial DCN and respiration [30], we expected that animals with conditional loss of *En1/2* in excitatory cerebellar neurons would show altered eupneic respiratory rhythmicity compared to controls. This study confirmed our expectations in that mutant animals had significantly increased intrinsic respiratory rhythmicity, as evidenced by a decrease in CV2. These results mirrored Liu *et al.*, where Purkinje cell silencing also caused decreased CV2 [28]. As predicted, we also found no difference between mutant *Atoh1-En1/2* animals and their control littermates in average respiratory rate. However, this present study diverged from expectations about the distribution of IRIs as measured by CV. While we hypothesized no change in IRI variability or CV, we found that mutant *Atoh1-En1/2* animals exhibited decreased overall variability during eupnea. Finally, we found sex differences in all three response variables, with females having increased CV and CV2 values and decreased average respiratory rate as compared to males, irrespective of strain. A modest effect of sex was also found in Liu *et al.* for both respiratory rate and CV2, although no effect of sex was found for CV in that study. In humans, there is broad evidence of physiological differences in respiration between the sexes, but these have been mainly attributed to the average size difference between male and female bodies [29]. For example, smaller lung size in females may lead to smaller tidal volume than males, requiring a faster respiratory rate to compensate. In contrast, our results showed a slower respiratory rate in females, but we cannot say whether this was due to differences in tidal volume because it was not measured. In a study in developing rats (neonatal to young adult, P10-P90), Holley *et al.* found that both age and sex affected respiratory responses to hypoxia, hypercapnia, and normoxia [21]. Most relevant to this study, they found that male P12-13 rats (in the postnatal "sensitive period") had significantly decreased minute ventilation and tidal volume in normoxic conditions as compared to females. This also does not account for the sex differences in CV or CV2, but these results certainly indicate that future studies of eupneic respiration should continue to include sex as factor. In addition, given that the *Atoh1-En1/2* CKO strain affects cerebellar development specifically, future research with this model should repeat whole-body unrestrained plethysmography at different developmental time points to understand how mutant respiratory response changes with age and sex.

The proposed role of the cerebellum in coordinating respiratory and other orofacial movements has been investigated in both mice and humans, and the vast reciprocal connections between the cerebellum and brainstem provide a putative anatomical substrate [42, 4, 18, 5, 30, 62]. Inappropriate coordination of swallowing and respiration can have detrimental effects, as in aspiration pneumonia due to dysphagia in patients with cerebellar ataxia [42]. In our recently published paper by, we sought to artificially “restore” appropriate cerebellar coordination by introducing intrinsic rhythmicity into the respiratory sequence of mutant animals with Purkinje cell silencing [28]. In this model, we altered the true mutant respiratory sequence *in silico* by lengthening every 10th IRI by 50% (about 75 ms). With this change, there was no longer a significant difference between the CV2 of the altered respiratory sequence and that of control animals. In addition, this alteration did not introduce a significant difference in CV between mutants and controls. This suggested that the CRB may have a role in altering the length of select respiratory intervals, and we speculated that this pause in respiration could be induced by the CRB to allow time for swallowing. Future studies should take care to include additional behavioral measures such as licking, swallowing, and whisking in order to develop a more complete understanding. These studies could also dissect breathing at rest from sniffing behaviors, as this present work considered eupneic respiration as whole.

The *Atoh1-En1/2* CKO strain provides unique insight into cerebellar temporal coordination. The recent consensus paper by Bareš *et al.* emphasized three important aspects of the cerebellum which make it suited for its timekeeping role: microcircuitry, neuronal density, and vast extracerebellar interconnectivity [1]. Willett *et al.* reported that this mutant model has normal neuronal density, despite its proportionally decreased neuronal number in certain hypoplastic lobules [53]. It is generally accepted that PCs in the cerebellar cortex project to the DCN which is anatomically closest to them [26], and accordingly, Willett *et al.* showed that PCs in the affected hypoplastic lobules projected to the medial and intermediate DCN, which were also reduced in size. This suggests that while these structures are reduced in size, the projection patterns from PCs to DCN are likely maintained. Therefore, we suggest that the altered respiratory rhythmicity is due to an error in the third essential aspect of cerebellar design – its highly organized and extensive extracerebellar interconnectivity.

While microcircuitry remains largely unchanged throughout the healthy cerebellar cortex, the discovery of molecular parasagittal stripes and functional connectivity mapping have revealed that the cerebellum is organized into spatially distinct zones [37, 6]. In 2008, Sillitoe *et al.* reported that *Engrailed 1* and *2* are not only required for normal foliation and molecular patterning of the cerebellum, but that they are also involved in mossy fiber afferent targeting [47]. This paper also suggested that morphological defects do not necessarily imply circuit defects. Instead, they urged that a better predictor of improper circuitry is an aberrant molecular pattern. In a mouse strain with conditional loss of *En1/2* in the whole cerebellum except for PCs, foliation defects were present but the molecular pattern and circuitry were intact. Thus the authors posited that PC expression of *En1/2* was required for guiding mossy fiber afferents. It also important to note that while the

Atoh1-En1/2 CKO strain affects cerebellar granule cells and the eCN, it is unknown what effect conditional loss of *En1/2* in other rhombic lip-lineage structures, such as certain pre-cerebellar nuclei, may have on respiration. Although the recent study by Liu *et al.* showed that CRB-specific mutations are sufficient to alter the CV2 of eupneic respiration [28], it does not preclude the possibility that extracerebellar loss of *En1/2* could also influence the CV and CV2 of eupneic respiration. I suggest that future research focus on further characterizing the *Atoh1-En1/2* CKO model, evaluating potential changes to the cerebellar molecular pattern and circuitry, as well as investigating potentially affected extracerebellar structures, to better understand how these factors may influence the cerebellar role in eupneic respiratory patterns.

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Vita

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