INVESTIGATING THE TRANSITION FROM PASSIVE TO
ACTIVE EXPIRATION IN RODENTS

by

Sarah Eleanor Maclay Jenkin

B.Sc., The University of Guelph, 2009
M.Sc., The University of Toronto, 2011

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in
THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES
(Zoology)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

December 2016

© Sarah Eleanor Maclay Jenkin, 2016
Abstract

Under eupneic conditions, the breathing cycle is composed of three phases: inspiration (I), expiratory braking (E1) and a secondary expiratory phase (E2) during which there may be passive expiration and potentially an expiratory pause. Thus, inspiration is active and expiration is passive. With elevated respiratory drive, the abdominal muscles are recruited to exhale air forcefully from the lungs, producing active expiration (AE). Breathing is a highly integrated response, dependent on peripheral and central feedback. What is required to successfully transition from a passive to an active expiration is still unknown. The goal of this thesis was to determine what stimuli are required to recruit AE using a comparative approach. We determined which stimuli recruit AE, where within the breathing cycle AE occurs (early-expiration or late-expiration), and how the breathing cycle accommodates this new phase. Three different rat preparations (in vivo, in situ, and in vitro) were used to determine how anesthesia, afferent vagal feedback and peripheral and central chemoreceptors contribute to the production of AE.

Hypoxia, anesthesia and afferent vagal feedback inhibited AE, while hypercapnia recruited AE. Central chemoreceptors were necessary to produce AE in vivo, but were insufficient to recruit AE in vitro. Within the pons, the Kölliker-Fuse actively inhibited the onset of AE activity, but did not directly initiate AE. Hypercapnia increased tidal volume (V_T) and produced AE in all rodent species studied. However, there were differences in what CO₂ levels recruited AE, and where AE occurred within the breathing cycle. Most species recruited a late-expiratory AE, while agoutis, with elevated breathing frequencies and high dynamic compliance, recruited an early-expiratory AE. While AE can recruit the expiratory reserve volume to enhance V_T in all species studied, the early-expiratory AE may enhance airflow to overcome an expiratory time...
constant that approaches the full expiratory phase duration (T_E). This thesis highlights that AE is a flexible process, which can influence the breathing response in multiple ways (control of V_T vs. control of T_E). This suggests that the removal of the inhibitory inputs to the expiratory neural regions likely occurs through different mechanisms under different circumstances.
Preface

Thesis represents the work completed by Sarah E. M. Jenkin, while under the supervision of Dr. Bill Milsom. The overall design of the research program was due to combined input from both Sarah E. M. Jenkin and Dr. Bill Milsom, in addition to feedback from the supervisory committee (Dr. Doug Altshuler, Dr. Bob Shadwick, and Dr. Bill Sheel).

Sarah E. M. Jenkin completed Chapter 3 while working with Dr. Daniel B. Zoccal at the Ararquara campus of São Paulo State University, Brazil. Sarah E. M. Jenkin was responsible for all of the experimental surgeries and the data collection. Dr. Marian Bassi assisted with the generation of the brain sections and fluorescence images. Sarah E. M. Jenkin and Dr. Zoccal worked on the data analysis and the figure generation. Sarah E.M. Jenkin, Dr. Daniel Zoccal and Dr. Bill Milsom contributed to the writing. This work was funded in part by Dr. Bill Milsom and by Dr. Daniel Zoccal. A version of Chapter 2 has been submitted for publication (submitted August 2016).

The data collection of Chapter 4 would not have been possible without the assistance of Dr. Angelina Fong, who instructed Sarah E. M. Jenkin on the methodology of the in vitro brainstem spinal-cord preparation. Sarah E. M. Jenkin completed all of the data collection and analysis.

A number of species used in Chapter 5 and 7 were studied while at the Jaboticabal Campus of São Paulo State University, Brazil. Dr. Andre Escobar anesthetized and cared for these animals throughout the data collection. Dr. Luciane Gargaglione and Dr. Glauber de Silva were critical in ensuring the availability of the animals and assisting with data collection.

All experiments were performed with approval from the University of British Columbia Animal Care Committee (A13-0025), under the guidelines of the Canadian Council for Animal
Care (CCAC). While in Araraquara, experimental procedures followed the guidelines of the Brazilian National Council for Animal Experimentation Control (CONCEA) and of the US National Institutes of Health (NIH, publication no. 85-23, 1996). All experimental protocols were authorized by the Local Ethical Committee in Animal Experimentation of the School of Dentistry of Araraquara (protocol 18/2014).
Chapter 2: Hypercapnia and vagotomy recruit late expiratory abdominal muscle activity to enhance tidal volume, while hypoxia does not

2.1 Introduction

2.2 Methods

2.2.1 Experimental animals

2.2.2 Surgical procedure

2.2.3 EMG lead placement

2.2.4 Experiment One: effect of hypercapnia on the recruitment of AE in vivo

2.2.5 Experiment Two: effect of vagotomy on the recruitment of AE in vivo

2.2.6 Experiment Three: effect of hypoxia on the recruitment of AE in vivo
2.2.7 Experiment Four: effect of anesthetic on the recruitment of AE in vivo ................ 15

2.2.8 Analysis of traces ........................................................................................................ 15

2.2.9 Statistical analysis ........................................................................................................ 16

2.3 Results ..................................................................................................................................... 17

2.3.1 Hypercapnia .................................................................................................................. 17

2.3.2 Hypoxia ......................................................................................................................... 19

2.4 Discussion ............................................................................................................................. 19

2.4.1 Caveats .......................................................................................................................... 19

2.4.2 Recruitment of the abdominal muscles ....................................................................... 21

2.4.3 Recruitment of the intercostal muscles ...................................................................... 27

2.4.4 Control of the phases of the breathing cycle ............................................................... 29

2.4.5 General conclusions ...................................................................................................... 30

Chapter 3: The Kölliker-Fuse Nucleus acts as a timekeeper for late-expiratory abdominal
activity ................................................................................................................................. 46

3.1 Introduction .......................................................................................................................... 46

3.2 Methods ............................................................................................................................... 48

3.2.1 Experimental animals ................................................................................................. 49

3.2.2 The in situ working heart brain preparation ................................................................ 49

3.2.3 Data acquisition and analysis ....................................................................................... 50

3.2.4 Activation of peripheral and central chemoreceptors ............................................... 50

3.2.5 Microinjections into the Kölliker-Fuse ....................................................................... 51

3.2.6 Histology ...................................................................................................................... 52

3.2.7 Statistical analysis ......................................................................................................... 52
3.3 Results................................................................................................................................. 52
  3.3.1 Effect of isoguvacine microinjections in the KF on baseline activities .................. 53
  3.3.2 Effect of KF inhibition on the peripheral O₂ chemoreflex activation ................. 53
  3.3.3 Effect of KF inhibition via isoguvacine on the hypercapnic response .............. 54
3.4 Discussion .......................................................................................................................... 55
  3.4.1 The role of the KF in the control of the expiratory phase during eupnea .......... 56
  3.4.2 The role of the KF in the control of late-E activity during peripheral and central chemoreflex activation ........................................................................................................ 57
  3.4.3 Conclusion..................................................................................................................... 60

Chapter 4: The hypercapnic ventilatory response in neonatal rats: the balance between factors intrinsic and extrinsic to the medulla ............................................................................. 69

  4.1 Introduction ....................................................................................................................... 69

  4.2 Methods ............................................................................................................................. 71
    4.2.1 Experimental animals ................................................................................................. 71
    4.2.2 The in vitro brainstem spinal cord preparation .......................................................... 71
    4.2.3 Data and statistical analysis ...................................................................................... 72

  4.3 Results .............................................................................................................................. 73
    4.3.1 Effect of CO₂ on burst area (indicative of tidal volume) ......................................... 73
    4.3.2 Effect of CO₂ on fictive breathing frequency ............................................................. 74
    4.3.3 Effect of CO₂ on inspiratory and expiratory time ...................................................... 75
    4.3.4 The effect of age and the pons on respiratory – related motor output .................... 76
    4.3.5 The effect of CO₂, age and the pons on C4-T11 burst coordination ......................... 76

  4.4 Discussion ......................................................................................................................... 76
4.4.1 Effect of CO$_2$ on burst area (fictive V$_T$) ................................................................. 77
4.4.2 Effect of CO$_2$ on fictive breathing frequency ............................................................. 78
4.4.3 The effect of CO$_2$, age and the pons on C4-T11 burst coordination ............................. 79
4.4.4 Conclusion .................................................................................................................. 80

Chapter 5: Respiratory muscle recruitment under hypercapnic conditions: a comparative
analysis in rodents ............................................................................................................... 89

5.1 Introduction ............................................................................................................... 89

5.2 Methods .................................................................................................................. 90
5.2.1 Experimental animals ............................................................................................. 91
5.2.2 Surgical procedure ................................................................................................. 91
5.2.3 Experimental procedure .......................................................................................... 92
5.2.4 Data analysis ........................................................................................................ 93
  5.2.4.1 Tidal volume and breathing frequency ............................................................. 93
  5.2.4.2 EMG trace analysis ........................................................................................... 93
5.2.5 Statistical analysis ................................................................................................. 94

5.3 Results .................................................................................................................... 94
5.3.1 Breathing frequency and tidal volume changes with progressive hypercapnia ....... 95
5.3.2 Inspiratory and expiratory phase changes with progressive hypercapnia .............. 96
5.3.3 Active expiation occurs under hypercapnic conditions ........................................... 96

5.4 Discussion .............................................................................................................. 97
5.4.1 Rodents do not exhibit AE at rest ........................................................................... 97
5.4.2 All rodents exhibit a three phase breathing cycle at rest ......................................... 98
5.4.3 The effect of progressive hypercapnia on the breathing cycle ................................. 99
Chapter 6: Respiratory muscle recruitment under hypoxic conditions: a comparative analysis in rodents

6.1 Introduction

6.2 Methods

6.2.1 Experimental animals

6.2.2 Surgical procedure

6.2.3 EMG lead placement

6.2.4 Hypoxic exposure

6.2.5 Data analysis

6.2.5.1 Measuring tidal volume and breathing frequency

6.2.5.2 EMG analysis

6.2.6 Statistical analysis

6.3 Results

6.4 Discussion

Chapter 7: Pulmonary mechanics and the presence of AE in rodents: a comparative analysis

7.1 Introduction

7.2 Methods

7.2.1 Experimental animals
7.2.2 Experimental procedure ................................................................. 144
7.2.3 Measuring lung volumes ................................................................. 144
7.2.4 Measuring static compliance ......................................................... 144
7.2.5 Measuring dynamic compliance .................................................... 145

7.3 Results ............................................................................................. 146
    7.3.1 Static pressure-volume measurements ....................................... 146
    7.3.2 Dynamic pressure-volume measurements ................................. 147

7.4 Discussion ...................................................................................... 148

Chapter 8: Conclusion ......................................................................... 169
    8.1 Respiratory stimuli modify the breathing cycle differently .......... 169
    8.2 The neural sites that generate breathing ...................................... 169
    8.3 The critical role of the pons and afferent vagal feedback in modifying the breathing response ................................................................. 171
    8.4 The importance of disinhibition in the recruitment of the respiratory muscles of the rat ................................................................. 172
    8.5 The role of the Kölliker-Fuse and afferent vagal feedback ............. 175
    8.6 Hypercapnia and hypoxia modify the respiratory phases of rodents differently ................................................................. 177
    8.7 Strengths and limitations of the thesis research ............................ 179
    8.8 Future directions ........................................................................... 180
    8.9 Significance of research ................................................................. 181

Bibliography .......................................................................................... 183
List of Tables

Table 2.1 The impact of tonic and phasic vagal feedback on the recruitment of active expiration.

................................................................. 45

Table 4.1 The inspiratory (T_I) and expiratory (T_E) for both the C4 motor nerve and T11 motor nerve bursts.

................................................................. 87

Table 5.1 Breathing parameters of 10 rodent species under eupneic conditions.

................................................................. 121

Table 5.2 Comparison of T_E/T_TOT between present study and Boggs and Tenney (1984).

................................................................. 122

Table 7.1 Volumes of the components of the respiratory system for nine rodent species.

................................................................. 166

Table 7.2 Static compliance for nine rodent species.

................................................................. 167

Table 7.3 Dynamic compliance and resistance for eight rodent species.

................................................................. 168
List of Figures

Figure 1.1 Schematic diagram of the respiratory muscles. ................................................................. 7
Figure 1.2 Schematic diagram of the dorsal and lateral view of the mammalian brainstem, highlighting key respiratory regions within the pons and medulla. ........................................... 8
Figure 1.3 Schematic diagram of the peripheral and central feedback that control and regulate the breathing response. ........................................................................................................ 9
Figure 1.4 Schematic representation of the abdominal EMG activity of A) horses, B) dogs and C) the short tailed opossum under eupneic conditions. ............................................................... 10
Figure 2.1 Representative trace of the durations and offsets of different muscles. ....................... 33
Figure 2.2 Effect of progressive hypercapnia on the tidal volume (measured with impedance; V) and breathing frequency (min\(^{-1}\)) of unanesthetized (A, B) and anesthetized rats (C, D) with vagi intact, and for vagotomized rats (E, F). ......................................................................................... 35
Figure 2.3 Effect of progressive hypercapnia on the duration of the inspiratory, expiratory braking and second expiratory phases. .......................................................................................... 37
Figure 2.4 Effect of progressive hypercapnia on the duration and the coordination of the respiratory muscles. .......................................................................................................................... 39
Figure 2.5 Effect of progressive hypoxia on the tidal volume (measured with impedance; V) and breathing frequency (min\(^{-1}\)) of unanesthetized (A, B) and anesthetized rats (C, D). ................. 40
Figure 2.6 Effect of progressive hypoxia on the duration of the inspiratory, expiratory braking and second expiratory phases........................................................................................................... 41
Figure 2.7 Effect of progressive hypoxia on the duration and the coordination of the respiratory muscles.......................................................................................................................... 43
Figure 2.8 Representative traces of the respiratory muscle recruitment of bilaterally vagotomized rat across progressive hypercapnia. .......................................................... 44

Figure 3.1 Raw and integrated (∫) recordings of the abdominal (AbN), cervical vagus (cVN), and phrenic nerve (PN) activities of an in situ preparation, representative from the group, illustrating the pattern of respiratory changes elicited by unilateral microinjection of glutamate (10mM) into the KF (arrow).................................................................................................................................................. 61

Figure 3.2 Panel A: Bright-field (top) and fluorescent (bottom) microscope images of brainstem coronal sections of a representative in situ preparation, showing the injection site of isoguvacine into the KF. Panel B: schematic representation of all injection (N=13) sites into the KF.

Abbreviations: DLL – dorsal nucleus of the lateral lemniscus; scp – superior cerebellar peduncle; s5 – sensory root of trigeminal nerve.......................................................... 62

Figure 3.3 Raw and integrated (∫) recordings of the abdominal (AbN), cervical vagus (cVN), and phrenic nerve (PN) activities of an in situ preparation, representative from the group, showing the respiratory responses to bilateral injections of the isoguvacine (arrows) into the KF. ............ 64

Figure 3.4 Raw and integrated (∫) recordings of cervical vagus (cVN), abdominal (AbN) and phrenic nerve (PN) activities of an in situ preparation, representative from the group, showing the increase in breathing frequency upon peripheral chemoreflex activation (with KCN, arrows) before and after the inhibition of KF with bilateral microinjections of isoguvacine (10 mM, ISO arrow)........................................................... 66

Figure 3.5 The raw and integrated (∫) trace of abdominal (AbN), cervical vagus (cVN), and phrenic nerve (PN) activities from an in situ preparation, representative of the group, showing the pattern of respiratory motor activities under baseline conditions (5% CO₂, 95% O₂) and under
hypercapnic conditions (8% CO$_2$, 92% CO$_2$) before, after KF inhibition with isoguvacine (10 mM) and after recovery from the KF inhibition. ................................................................. 68

Figure 4.1 Effect of age and CO$_2$ levels on the fictive tidal volume of C4 and T11 motor nerve output from ponto-medullary and medullary preparations. .......................................................... 82

Figure 4.2 Effect of age and CO$_2$ levels on the fictive breathing frequency of C4 and T11 motor nerve output from ponto-medullary and medullary preparations. .......................................................... 84

Figure 4.3 Effect of age and CO$_2$ levels on the onset and duration of the C4 and T11 motor nerve activity.......................................................................................................................... 86

Figure 5.1 Representative traces of breathing and the respiratory muscles. Trace of breathing (measured with impedance, upward inflection represents an inspiration), the raw and integrated diaphragm (DIA$_m$) and abdominal muscles (ABS$_m$) are presented...................................................... 105

Figure 5.2 Representative trace of the occasional active expiration exhibited by gerbils and mice.................................................................................................................................................. 106

Figure 5.3 Comparing the inspiratory duration (T$_I$) to the total expiratory phase duration (T$_E$) among a number of rodent species under eupneic conditions. .............................................. 107

Figure 5.4 Relationship between breathing frequency (min$^{-1}$) and the inspiratory duty cycle (T$_I$/T$_{TOT}$)...................................................................................................................... 108

Figure 5.5 Comparing the expiratory braking phase duration (T$_{E1}$) and the second expiratory phase duration (T$_{E2}$) among a number of rodent species under eupneic conditions........... 109

Figure 5.6 Relationship between breathing frequency (min$^{-1}$) and expiratory braking (T$_{E1}$/T$_{TOT}$) and second expiratory phase duty cycle (T$_{E2}$/T$_{TOT}$)........................................................... 110

Figure 5.7 Effect of progressive hypercapnia on the tidal volume and breathing frequency of 10 rodent species.................................................................................................................. 112
Figure 5.8 Effect of progressive hypercapnia on the duration of the breathing cycle phases of 10 rodent species ................................................................. 114
Figure 5.9 Effect of progressive hypercapnia on $V_T/T_1$ of 10 rodent species ....................... 116
Figure 5.10 Effect of progressive hypercapnia on the duty cycles of the breathing phases of 10 rodent species ................................................................. 118
Figure 5.11 Average duty cycles for expiration ($T_E$), expiratory braking ($T_{E1}$) and active expiration ($T_{AE}$) exhibited by 10 rodent species ......................................................... 119
Figure 5.12 Representative traces of the different breathing patterns generated when active expiration is present ........................................................ 120

Figure 6.1 Effect of progressive hypoxia on the tidal volume and breathing frequency of seven rodent species ................................................................. 133
Figure 6.2 Effect of progressive hypoxia on the duration of the breathing cycle phases of seven rodent species ................................................................. 135
Figure 6.3 Effect of progressive hypoxia on $V_T/T_1$ of seven rodent species ..................... 137
Figure 6.4 Effect of progressive hypoxia on the ratio of inspiration to expiration ($I/E$) of seven rodent species ................................................................. 139
Figure 6.5 Effect of progressive hypoxia on the duty cycles of the breathing phases of seven rodents species ................................................................. 141

Figure 7.1 Schematic representation of typical mammalian static pressure-volume deflation curves ................................................................. 153
Figure 7.2 Schematic diagrams illustrating how measurements of dynamic compliance and resistance were obtained from the pressure-volume curves ......................................... 154
Figure 7.3  Schematic diagram illustrating how the passive expiratory time constant was obtained from a flow-volume loop. ................................................................. 155

Figure 7.4 Average expiratory reserve volume as a percentage of the vital capacity of nine rodent species. ............................................................ 156

Figure 7.5  Relationship between body mass (kg) and vital capacity (mL). ................................ 157

Figure 7.6 Average static compliance curves of nine rodent species. ........................................... 159

Figure 7.7  Effect of increasing tidal volume on dynamic compliance for eight rodent species.
.............................................................................................................. 161

Figure 7.8  Effect of increasing tidal volume on resistance for eight rodent species. .............. 162

Figure 7.9  Comparison of the passive expiratory time constant (τE), expiratory braking phase duration (T_{E1}) and total expiratory phase duration (T_{E}) for eight rodent species. ...................... 165
List of Abbreviations

ABS$_{m}$ – abdominal muscle activity
AE – active expiration
BötC – Bötzinger Complex
C – compliance
cm H$_2$O – centimeters of water
DIA$_{m}$ – diaphragm muscle activity
E – expiratory phase
E1 – expiratory baking phase or first phase expiration
E2 – second phase of expiration
ERV – expiratory reserve volume
$f_R$ – breathing frequency (breaths/min)
I – Inspiratory phase
INT$_{m}$ – intercostal muscle activity
IRV – inspiratory reserve volume
KF – Kölliker-Fuse
min – minute
mL – milliliter
msec – millisecond
preBötC – preBötzinger Complex
R – resistance
RTN/pFRG - retrotrapezoid nucleus/ parafacial respiratory group
sec – second

\( T_{AE}/T_{TOT} \) – active expiration duty cycle

\( T_E \) – total expiratory duration

\( T_{E1} \) – expiratory braking duration

\( T_{E1}/T_{TOT} \) – expiratory braking duty cycle

\( T_{E2} \) – second expiratory phase duration

\( T_{E2}/T_{TOT} \) – second expiratory phase duty cycle

\( T_1 \) – Inspiratory duration (sec)

\( T_I/T_{TOT} \) – inspiratory duty cycle

\( T_{TOT} \) – duration of the total breathing cycle time

\( \dot{V}_E \) – ventilation

\( VC \) – vital capacity

\( V_T \) – tidal volume

\( \tau_E \) – passive expiratory time constant
Glossary

\( \tau \) (Tau; sec) represents a time constant. The expiratory time constant represent the time it takes for the respiratory system to passively deflate. The expiratory time constant was measured by dividing the tidal volume by the maximal expiratory flow.

**Compliance** (mL cm H2O\(^{-1}\)) is the change in volume for a given change in pressure. If the respiratory system is highly compliant, it will expand greatly with very little pressure change. It is the inverse of stiffness and elastance. Compliance can be calculated from a system with no airflow (static compliance) or from a system that is undergoing airflow (dynamic compliance).

**Resistance** (cm H\(_2\)O mL\(^{-1}\) min\(^{-1}\)) is measured as the difference in pressure between two points of isovolume of a dynamic breathing cycle (pressure-volume loop), divided by the flow (Mead, 1961). The resistance of the respiratory system is dependent on the relative change in pressure between air entering and leaving the lungs and the flow rate.

**Eupnea** is a term to describe a “normal” or unlabored breathing pattern. It is considered synonymous with quiet breathing or resting breathing.

**Duty cycle** is a ratio of the duration of an active period to the total cycle time.
Acknowledgements

First and foremost, I need to thank Bill Milsom for all of his support, encouragement and advice over the last five years. You helped me navigate new ideas and new methodologies to create a pretty exciting thesis. Thank you to my committee (Doug, Bob and Bill) for the multiple committee meetings, guidance and support.

Thank you to Charissa, Yvonne, Sabine, Nicki, Michelle, Julia, Anne and Ryan. From dancing with Oak after tear-filled breakdowns and riding double decker buses through Edinburgh to hiking through snow covered mountains in Manning Park. You all made my time here so much better.

To the Hen House: Erica, Michelle, Emily, Katelyn, Libby and Ellen. Thank you for giving me an escape from work, for the clothing swaps, the decorating parties, the endless stream of dead and living plants, and the true crime stories. I am forever grateful that you were brought into my life, even if just for a short while.

To Agnes Lacombe, thank you for encouraging me to grow into an enthusiastic and driven teacher. To Jim Cooke, thank you for you support of not only my teaching but also of my own research. I constantly think back on your advice in navigating the world of graduate school.

Thank you to Angelina Fong and Tara Bautista, two outstanding women who helped me navigate the in vitro and in situ preparations.

Over the summer of 2015, I went to Brazil to battle with rodents of unusual size and conquered the frightening monster that is the in situ preparation. Thank you to Glauber and Dr. André Escobar for all of your help with the large rodent EMG study. To fixing EMG leads to suddenly performing CPR on a 30 kg Capybara, the data I collected while in Jabuticabal was
certainly due to your efforts. There are not enough words to thank Dr. Daniel Zoccal for his help with our study on the Kölliker-Fuse. Daniel, thank you for providing me an environment where I felt unconditionally supported. I was only able to complete such a challenging project in such a short time because of you and your encouragement.

To my family and friends that maintained my sanity and provided me with a constant source of encouragement. Poppy and Mummie, thank you for editing many thesis chapter drafts, supporting my every decision, celebrating every victory, and loving me through the daily challenges and failures that go along with being a graduate student. To Emma, Dale, Heath and Adelaide thank you for showing me love and support and reminding me that jobs can be crap, but that things will be alright if you can have a fabulous family to come home to after a terrible day at work. To Pooks, thank you for being a welcoming face whenever I would return home. I’ll miss you forever and always.

Finally, to my true partner in crime - Jacob. I can honestly say that I would not have survived my PhD without you. You are the light in all the darkness, the antidote to the poison that I drink, the butter to my gluten free bread, the breath to my life. You dragged me to this finish line, ensuring I never gave up on myself and I can’t wait to end this chapter and move on to bigger and better things.
Chapter 1: Introduction

Breathing is a highly integrated behavior that is critical for survival. For the appropriate movement of airflow, inspiration - the process of bringing air into the lungs - and expiration - the process of exhaling air from the lungs - are dependent on the coordinated contractions of a number of respiratory muscles (Figure 1.1). Although the mammalian breathing cycle can be superficially divided into these two phases (inspiration and expiration), it is in fact composed of three phases under eupneic conditions (Richter, 1996). The first of these three phases consists of an active inspiration (I), where the diaphragm contracts and air is drawn into the lungs.

Expiration is composed of two phases. The first phase (E1) is defined by post-inspiratory (post-I) activity, which slows the relaxation of the inspiratory muscles. This is known as expiratory braking, and it is thought to prolong gas exchange (Richter and Smith, 2014). Under eupneic conditions, the second and final phase of expiration (E2) can be defined by passive expiration and/or a ventilatory pause where there is no muscle contraction and no airflow (Richter, 1996; Feldman and McCrimmon, 2003). This produces a breathing cycle where inspiration is always an active process and expiration is typically a passive process (Iscoe, 1998; Jenkin and Milsom, 2014 for review). Over the last 25 years, there has been substantive interest in understanding the control of the inspiratory phase of the mammalian breathing cycle. As a result, expiration became the forgotten phase of the breathing cycle, considered less important than its active inspiratory counterpart. The lack of interest in expiration is quite surprising, as a mammal’s drive to breathe is not dictated by the need for oxygen, but instead by the need to release carbon dioxide from our system, highlighting the importance of expiration in driving the breathing response of mammals (see Ultsch, 1996 for review). In addition, the system has a great capacity
to transition, when needed, from a passive to an active expiration (see Feldman et al., 2013 for review).

All of the respiratory muscle contractions that power the mammalian aspiration pump are under the control of highly organized and connected groups of neurons (Figure 1.2). These neurons are highly dynamic, as they are designed to adjust to the demands of the environment, in addition to any peripheral or descending feedback, to ensure appropriate levels of ventilation are maintained. The currently accepted hypothesis for the control of breathing is that a region of the medulla, known as the ventral respiratory column (VRC), controls the rhythmic act of eupneic breathing. Within the VRC are two central pattern generators (CPGs): the preBötzing Der Complex (preBötC) and the Bötzinger Complex (BötC). The preBötC is thought to be the inspiratory oscillator, critical and sufficient for the generation of inspiratory rhythmic activity (Smith et al., 1991). Neurons from the preBötC are active during inspiration (I) and are thus termed ‘I’ neurons. Caudal to the preBötC lies the BötC, which contains expiratory (E) neurons. Within the BötC there are neurons that fire during the first portion of expiration (E1 or post-I neurons), and neurons that fire during the end of expiration (E2 or augmenting expiratory (aug-E) neurons) (Smith et al., 2009). These I, E1 and E2 neurons project to, and synapse on, the cranial and spinal motor nerves to power the appropriate respiratory muscles at the appropriate time (Smith et al., 2009).

When respiratory drive is elevated, expiration often transitions from passive to active. During an active expiration (AE), the abdominal and internal intercostal muscles can be recruited to force air out of the lungs (see Feldman et al., 2013 for review). This activity appears to be dependent on the neuronal activity of a separate region of the medulla, known as the retrotrapezoid nucleus/parafacial respiratory group (RTN/pFRG). The RTN is a highly
chemosensitive group of cells that lie next to the pFRG (Guyenet et al., 2009). The current hypothesis is that the pFRG is the expiratory oscillator, critical for the production of expiratory abdominal muscle activity when respiratory drive is elevated (Onimaru et al., 1987; 1988, Onimaru and Homma, 2003; Jancewski and Feldman, 2006). Neurons from the pFRG tend to fire concurrently with the aug-E neurons of the BötC, and their activity is often defined as late expiratory (late-E) (Abdala et al., 2009). It should be mentioned that any increase in respiratory drive in vivo is not only due to stimulation of central chemoreceptors (like those of the RTN), but is also due to peripheral chemoreceptor stimulation (Lahiri and Forster, 2003). To date, little work has investigated the role of peripheral chemoreceptors in generating AE.

While these CPGs generate breathing rhythm, both inspiration and expiration are controlled by a number of integrated feedback mechanisms throughout the body (Figure 1.3). The following sections will focus on the critical role of afferent vagal feedback and descending pontine influences on the transition between inspiration and expiration (Alheid et al., 2004; Molkov et al., 2013). The pons plays a role in coordinating the transition between the inspiratory and expiratory phases. Lumsden (1923) and Stella (1983) demonstrated that the pons and afferent vagal feedback act as a failsafe mechanism to produce eupnea (a normal breathing pattern), as it is only after both are inhibited that apneusis (a prolonged inspiration) occurs (Mörschel and Dutschmann, 2009). While the current view is that the pons plays a critical role in terminating inspiration, based on the cyclical nature of the breathing cycle, it is likely also critical in activating expiration. While Abdala and colleagues (2009) found that complete removal of the pons eliminates AE in vagotomized juvenile rat preparations and that AE was not recoverable even with an increased respiratory drive (CO₂), to date, there has been little work investigating how specific areas of the pons contribute to the generation of AE.
Afferent vagal feedback from stretch receptors in the lungs was discovered to play a role in the Hering-Breuer reflex, which ensures the lungs do not hyper-inflate or hyper-deflate (Breuer, 1868; Hering, 1868; see Moore, 1926). As afferent vagal feedback is critical for controlling the duration of the inspiration and expiration phases, bilateral vagotomy typically prolongs inspiration, resulting in a decrease in breathing frequency and an increase in tidal volume (Amphibians: Reid et al., 2000. Reptiles: Milsom, 1984; Milsom and Jones, 1980. Mammals: Fedorko et al., 1988; Harris and Milsom, 2001). To date, it remains unclear how the vagus nerve contributes to AE. Some studies report that vagal feedback is required to recruit expiratory abdominal muscle activity (Russel and Bishop, 1976; De Troyer and Ninane, 1987; Ainsworth et al., 1992; Yasuma et al., 1993), but is not required to recruit expiratory intercostal muscles (Ainsworth et al., 1992). In contrast, others report the presence of AE after vagotomy (De Troyer et al., 1989; Abdala et al., 2009; Lemes and Zoccal, 2014). The complexity of the role of afferent vagal feedback is influenced by the enhancement, reduction or complete removal of tonic and/or phasic feedback mechanisms (Bishop, 1962; Bishop and Bachofen, 1973; Fregosi et al., 1990; Leewers and Road, 1995).

To complicate matters, the concept that all mammals exhibit a passive expiration at rest is a great generalization, as there are known exceptions to this rule. For example, horses utilize a four-stage breathing pattern (Koterba et al., 1988; Koterba et al., 1995). In horses, the breathing cycle can be defined with the first expiratory phase beginning passively with the breaking of the diaphragm. During this phase, no expiratory muscles are recruited, but the diaphragm remains contracted to slow the release of air from the lungs. The second phase of expiration is active, requiring the contraction of the abdominal muscles, specifically the transversus abdominis (TA) muscle, to force the diaphragm cranially. The start of inspiration is passive; it depends on the
relaxation of the abdominal muscles and allows the diaphragm to return to a restful state.

Inspiration then becomes active, as the inspiratory muscles (the diaphragm and the internal intercostal muscles) contract (Koterba et al., 1988; Koterba et al., 1995; De Troyer and Loring, 2011). Based on the presence of the passive expiration phase, it is unlikely that the active component of expiration plays a role in speeding up expiration, but rather contributes to an increased tidal volume of the following inspiration (De Troyer and Loring, 2011). Both anesthetized (Gilmatin et al., 1987) and unanesthetized (De Troyer et al., 1989) dogs also exhibit phasic TA muscle activity at rest. From the EMG traces recorded by De Troyer and colleagues (1989), this TA activity (representing AE) is present in the last two-thirds of the expiration phase. This is similar to the placement of AE found in the horse at rest, and likely contributes to increasing inspiratory volume as well (Figure 1.4A,B). Reilly and White (2009) observed active abdominal muscle contractions during expiration in marsupials. The short-tailed opossum exhibits phasic abdominal muscle (internal and external oblique, rectus and transversus abdominis, and pyramidalis muscles) activity during expiration. Interestingly, the abdominal muscle activity occurs during the first half of the expiratory phase (Reilly and White, 2009; Reilly et al., 2009; 2010; Figure 1.4C). The placement of AE in the first half of the expiration cycle suggests it may play a role in decreasing expiratory time. To date, little work has been done to determine the commonality of active expiration under resting conditions among mammals, and if the relative placement of AE within the breathing cycle is a reflection of the mechanics of the respiratory system.

Throughout the literature, there are inconsistencies in the level of anesthetic used, the level of the respiratory drive used, the age of the animal, the type of species and the preparation used, leading to high degree of uncertainty about what is required to generate AE. Thus, the first
goal of my Doctoral Thesis was to explore the control of the transition from passive to active expiration. Specifically to determine what type of stimulus is required to produce an active expiration (Chapter 2), and how peripheral (Chapter 3) and central control (Chapter 4) contribute to the generation of active expiration. These questions were investigated using three different rat preparations; the in vivo, the in situ working heart brain preparation, and the in vitro brainstem-spinal cord preparations. The benefit of using these three preparations is that they have varying levels of stimulation present. The in vivo preparation has maximal levels of stimulation, and allows for observation of a system with fully intact peripheral and central feedback. The in situ preparation is decerebrate, lacking afferent vagal feedback to explore the importance of these mechanisms on breathing. Lastly, the in vitro preparation has the most reduced levels of stimulation, composed of only the functioning respiratory systems of the brainstem and connected circuitry passing the respiratory motor output to the spinal motor nerves, in the absence of any peripheral feedback mechanisms. Each preparation type also possesses the intact central medullary respiratory centers critical for the generation of both active inspiratory and expiratory neural activity. In addition, my thesis employed a comparative investigation of AE at rest, and where within the breathing cycle different rodent species utilize active expiration under hypercapnic (Chapter 5) and hypoxic (Chapter 6) condition as well as whether differences in the recruitment of AE were due to differences in the pulmonary mechanics of the species (Chapter 7).
Figure 1.1 Schematic diagram of the respiratory muscles. This figure represents the major thoracic muscles that power the movement of air during inspiration and expiration. The major inspiratory muscles are the diaphragm and the external intercostal muscles, while the major expiratory muscles are the internal intercostal muscles and certain abdominal muscles. The transversus abdominis muscle is considered to be the most critical abdominal muscle in powering an active expiration.
Figure 1.2 Schematic diagram of the dorsal and lateral view of the mammalian brainstem, highlighting key respiratory regions within the pons and medulla. This figure highlights the major neural regions that are critical to generating the inspiratory and expiratory motor output.

The Kölliker-Fuse nucleus is located in the pons. Within the medulla lies the retrotrapezoid nucleus/parafacial respiratory group (RTN/pFRG), which is critical for generating late expiratory abdominal activity, the Bötzinger Complex, which contains post inspiratory (E1) and augmenting expiratory (E2) neurons, the preBötzinger Complex, which contains the neurons that are sufficient to produce inspiratory activity, and the rostral ventral respiratory group (rVRG) and the caudal ventral respiratory group (cVRG) which contain the main bulbospinal inspiratory and expiratory neurons, respectively.
Figure 1.3 Schematic diagram of the peripheral and central feedback that control and regulate the breathing response. The overall breathing response is the product of a number of receptors and feedback mechanisms located throughout the body, from O$_2$, CO$_2$ and pH sensitive peripheral chemoreceptors of the carotid and aortic arches, mechanoreceptors of the rib cage, pulmonary stretch receptors of the lungs, CO$_2$ and pH chemoreceptors within the medulla, and higher brain centers (such as the hypothalamus and the cortex).
Figure 1.4 Schematic representation of the abdominal EMG activity of A) horses, B) dogs and C) the short tailed opossum under eupneic conditions. The black bars highlight the expiratory cycle, during which there is phasic abdominal muscle activity. Note that the abdominal muscle activity occurs at the end of the expiratory phase of horses and dogs, and at the beginning of the expiratory phase of the short-tailed opossum.
Chapter 2: Hypercapnia and vagotomy recruit late expiratory abdominal muscle activity to enhance tidal volume, while hypoxia does not

2.1 Introduction

At rest, the breathing cycle of most mammals is composed of three phases, an inspiratory phase and two expiratory phases (Cohen, 1979; Richter, 1996). A typical breathing cycle begins with an active inspiration (I), which is followed by the first expiratory phase (E1), characterized by a prolonged contraction of inspiratory muscles. This is known as expiratory braking and controls the rate at which air is exhaled from the lungs (Gautier et al., 1973; Feldman et al., 2013). The second phase of expiration can be defined by passive expiration and/or a ventilatory pause, where there is no muscle contraction and no airflow (E2) (Richter, 1996; Feldman and McCrimmon, 2003). Under resting conditions, this E2 phase continues until the following inspiration and the cycle repeats. At rest, expiration is passive; no muscle contraction is present to enhance expiratory airflow. When respiratory drive is elevated (as with exercise, stress, hypoxia, hypercapnia, etc.) the abdominal muscles and internal intercostal muscles can be recruited to exhale air forcefully from the lungs, producing an active expiration (AE) (De Troyer et al., 1989; Feldman et al., 2013). Understanding the triggers for AE and the nature of the transition between passive and active expiration is critical for our understanding of a number of respiratory disorders that impair expiration (such as chronic obstructive pulmonary disease (COPD)), in addition to furthering our understanding of the neuromuscular control of breathing.
2.2 Methods

All experiments were performed with approval from the University of British Columbia Animal Care Committee (A13-0025), under the guidelines of the Canadian Council for Animal Care (CCAC).

2.2.1 Experimental animals

Adult, female Sprague-Dawley rats were used in this study. All experimental trials were performed in vivo, and rats were either anesthetized (1.3-1.7 g/kg urethane, as described by Pagliardini et al., 2011; n=8) or unanesthetized. Unanesthetized rats were left with the vagi intact (n=8) or were bilaterally vagotomized (n=6).

2.2.2 Surgical procedure

Animals were anesthetized with ~2-4% isoflurane for the surgical placement of the electromyography (EMG) and impedance leads. A surgical level of anesthetic was determined when the toe pinch and corneal reflex were abolished. An overall analgesic (metacam 1ml/mg) was provided subcutaneously, and subcutaneous injections of lidocaine were provided at six small incision sites for the placement of electrodes into the diaphragm, the intercostal muscles, and the abdominal muscles, one on either side of the lateral thorax for the placement of impedance leads, and one on the back of the neck for a ground electrode.

2.2.3 EMG lead placement

Coated silver wires were used to record EMG activity of select inspiratory and expiratory muscles. A small portion of the wire coating was removed at the end; this uncoated end was
curved to form a hook that would secure the leads into the muscle. Coupled EMG silver wire electrodes were inserted into the diaphragm (DIAₘ), the intercostal muscles (INTₘ), and the abdominal muscles (ABSₘ) via a hypodermic 21G needle. Placement of the DIAₘ EMGs was towards the left side of the ventral surface, inserting the leads by sliding them under the ribs cage and advancing the needles and leads cranially until the hooks were inserted into the diaphragm. Placement of the INTₘ EMGs was within the lateral T11-T13 costal space. As the internal and external intercostal muscles of rats are very thin, the INTₘ EMGs would pass through both layers of muscles. As a result, when the INTₘ EMG activity was present during inspiration, it was considered to be recording activity from the external intercostal muscles, and when activity was present during expiration, it was considered to be recording activity from the internal intercostal muscles. Placement of the ABSₘ EMGs was towards the lateral side of the lower 3rd ventral abdominal surface, with care taken to reach through all the abdominal muscles layers without perforating the abdominal viscera. EMG grounds were placed in the back muscles of the animal by advancing the needles and leads into the visible muscle until the hook was imbedded securely. For each muscle group, the hook was sufficient to secure the leads for the duration of the experiment. The impedance leads were secured to shaved patches of skin on the lateral abdomen, as described by Harris (1998). The leads were fed subcutaneously to a small incision at the back of the neck to ensure the animals did not remove the leads throughout the duration of the experiment. After the successful placement of the leads, the rats were allowed to recover from the anesthetic and were placed in an experimental chamber and exposed to air (normoxic normocapnia) for at least 20 minutes to recover. Electrodes were connected to amplifiers (Dam50; World Precision Instruments, Inc.), filtered (0.1kHz-1kHz; as described by Navarrete-Opazo and Mitchell, 2014; Lemes and Zoccal, 2014) and activity was sampled at 2kHz and
stored on computer using a Windaq data acquisition system (D1200; DataQ Instruments, USA). The activity from each muscle was recorded simultaneously. The placement of the leads was verified by visual examination after each experiment.

2.2.4 **Experiment One: effect of hypercapnia on the recruitment of AE in vivo**

Following the 20-minute exposure to air, the rats (n=8) were exposed to progressive hypercapnia (0%, 2%, 4%, 6%, 8% and 10% CO₂ balanced with air, for 10 minutes at each exposure level).

2.2.5 **Experiment Two: effect of vagotomy on the recruitment of AE in vivo**

A group of rats (n=6) underwent bilateral cervical vagotomy while they were anesthetized for the placement of the EMG leads. After rats had recovered from the surgery, they were exposed to air conditions for 20 minutes to adjust to the chamber. Following this, the rats were exposed to progressive hypercapnia (0%, 2%, 4%, 6%, 8% and 10% CO₂ balanced with air, for 10 minutes at each exposure) as described above. Due to the extreme changes in breathing, the experiments were terminated once the animals exhibited active expiration.

2.2.6 **Experiment Three: effect of hypoxia on the recruitment of AE in vivo**

Following the 20-minute exposure to air, rats (n=8) were exposed to progressive levels of hypoxia (21%, 19%, 17%, 15%, 13%, 11% and 9% O₂ balanced with N₂), for 10 minutes at each exposure.
2.2.7 **Experiment Four: effect of anesthetic on the recruitment of AE in vivo**

Following the placement of the leads, rats (n=8) were anesthetized with 1.3-1.7 g/kg of urethane. Once anesthetized (determined by the lack of a toe pinch and corneal reflex) the rats were exposed to air for 20 minutes to recover, and then exposed to a progressive increase in hypercapnia, then returned to normoxic normocapnic conditions for a minimum of 20 minutes, followed by a progressive increase in hypoxia (the order of exposure was randomized).

2.2.8 **Analysis of traces**

Any DC offsets of the EMG signals were removed, the raw traces were rectified, and the absolute integral was calculated and smoothed (50 msec) for analysis (LabChart7, ADInstruments; as described by Lemes and Zoccal, 2014). The time points for the start and end of the diaphragm (DIA<sub>m</sub>), intercostal muscle (INT<sub>m</sub>), and abdominal muscle (ABS<sub>m</sub>) activity were recorded. For the DIA<sub>m</sub>, this was done to quantify the duration of the I phase (the contraction of the DIA<sub>m</sub> during the inspiration portion of the impedance trace), the E1 phase (the prolonged contraction of DIA<sub>m</sub> during the expiration portion of the impedance trace), and the E2 phase (the remaining expiratory portion of the impedance trace that had no DIA<sub>m</sub> activity) (See Figure 2.1A). The starting point of activity in both the INT<sub>m</sub> and ABS<sub>m</sub> relative to the DIA<sub>m</sub> starting point was calculated as each muscle’s “offset” time (See Figure 2.1B, C). The durations of the INT<sub>m</sub> and ABS<sub>m</sub> activity were determined. Due to variations in offset and duration of this activity from breath to breath, the offset and duration of activity in the INT<sub>m</sub> and ABS<sub>m</sub> for each breath were normalized. This was done by calculating the offset and duration as a percentage of the respective duration of the diaphragm activity for that breath (the sum of the diaphragm’s I, E1 and E2 durations). These values were averaged for all rats, and means ± S.E.M. were
calculated. The amount of activity for each muscle group was determined using the integral relative to baseline function in LabChart7 for ten consecutive breaths.

While tidal volume was never directly measured, the amplitude in the deflection in the impedance trace (V) over a 1-minute period was used as a correlate of tidal volume. The frequency of the impedance trace cycle over a 1-minute period was used to determine breathing frequency.

2.2.9 Statistical analysis

Differences in the amplitude of the impedance traces (representing tidal volume), frequency of the breathing cycle, and durations of the I, E1, and E2 phases of the DIA_m, the duration of the INT_m and ABS_m and the duration of the ventilatory pause of the INT_m and ABS_m between hypercapnic levels and between hypoxic levels were compared using a repeated measures ANOVA. A one-way ANOVA was used to determine any differences in the breathing frequency and tidal volume among unanesthetized rats with vagi intact and after bilateral vagotomy and anesthetized rats under normoxic normocapnic conditions. A repeated measures ANOVA was also used to compare the amount of EMG muscle activity for each muscle group between hypercapnic levels and between hypoxic levels. The comparisons were conducted in Sigma Stat 11 software (Dundas Software LTD). If normality or equal variance was not passed the ANOVA was calculated on ranks. Holm-Sidak or Tukey Tests were used as appropriate. Differences were considered significant when $p<0.05$. 
2.3 Results

2.3.1 Hypercapnia

In unanesthetized, vagus intact animals, progressive hypercapnia led to a significant increase in tidal volume ($p<0.001$) (Figure 2.2A). Breathing frequency increased progressively between 0% and 6% inspired CO$_2$ ($p<0.001$) but decreased again as the increase in tidal volume became significant ($p<0.001$) (Figure 2.2B). The increase in breathing frequency with hypercapnia was due to shortening of $T_{E1}$ (reduction of expiratory braking) ($p \leq 0.001$) (Figure 2.3B). There was a trend for $T_{E2}$ to decrease, reaching a minimum at 6% CO$_2$, only to increase again as tidal volume began to increase significantly (Figure 2.3C). Despite the large increases in tidal volume, there were no significant changes in $T_I$ (Figure 2.3A). As CO$_2$ levels increased, $INT_m$ activity appeared during $T_I$, becoming significantly shorter at 10% CO$_2$ compared to 2% CO$_2$ ($p=0.003$; Figure 2.4). At 8% CO$_2$, $ABS_m$ activity was recruited throughout $T_{E2}$. At this point tidal volume had increased by more than 250%. Thus at higher levels of inspired CO$_2$, the $INT_m$ assisted the $DIA_m$ during inspiration, there was little or no expiratory braking, and at 8% CO$_2$ and above $ABS_m$ activity appeared throughout most of $T_E$ and lasted into $T_I$. While not obvious in the mean data, the typical pattern in rats breathing high CO$_2$ was a four phase breathing cycle with the I, E1 and E2 phase of the $DIA_m$ being conserved and with AE starting at the end of the breathing cycle (late E2), and terminating early in the following inspiration.

Following anesthesia, the hypercapnic ventilatory response was greatly reduced compared to the response of unanesthetized rats. Tidal volume increased modestly but significantly (234% at the highest levels of inspired CO$_2$; $p<0.001$; Figure 2.2C). Breathing frequency, on the other hand, decreased significantly at severe levels of hypercapnia ($p \leq 0.003$ Figure 2.2D). $T_I$ remained relatively constant suggesting that inspiratory airflow increased.
(Figure 2.3D) but $T_{E2}$ was prolonged ($p\leq 0.001$; Figure 2.3F). Once again, the $\text{INT}_m$ were now active throughout $T_1$ continuing into $T_{E1}$ even in normoxic, normocapnic conditions (Figure 2.4). It should be noted that $\text{INT}_m$ activity was not observed in every animal even at 10% CO$_2$ (6 of 8 animals). This may have been due to differences in the placement of the leads (depth of the leads) or individual variation. $\text{ABS}_m$ activity, however, was never recruited (Figure 2.4). Thus during progressive hypercapnia under anesthesia tidal volume increased, the $\text{INT}_m$ assisted the $\text{DIA}_m$ during $T_1$ and during expiratory braking ($E_1$), which was mildly reduced, and there was no $\text{ABS}_m$ activity.

Following vagotomy tidal volume increased (466%) significantly from vagi intact rats ($p=0.037$) and breathing frequency decreased (to roughly 50%) compared to the vagi intact unanesthetized ($p=0.007$) and anesthetized rats ($p<0.001$; Figure 2.4). This was due to a lengthening of all phases of the respiratory cycle (Figure 2.4). $T_1$ more than doubled and the $\text{INT}_m$ were recruited throughout $T_1$ and into $T_{E1}$. $T_{E1}$ more than doubled and $T_{E2}$ more than tripled. Progressive hypercapnia still led to a significant increase in tidal volume ($p\leq 0.01$; Figure 2.2E) and breathing frequency decreased progressively, but not significantly (Figure 2.2F). The decrease in breathing frequency was due primarily to a lengthening of the expiratory pause ($T_{E2}$; Figure 2.3I). There were no significant changes in $T_1$ or in $T_{E1}$ at any level of inspired CO$_2$, however, at 6% CO$_2$, there was a significant increase in $T_{E2}$ ($p=0.012$; Figure 2.3G-I). As CO$_2$ levels increased, $\text{INT}_m$ activity was also recruited in late $T_{E2}$. The $\text{INT}_m$ activity appeared biphasic at this time suggestive of the recruitment of external intercostal muscles. $\text{ABS}_m$ activity was also recruited at 4% inspired CO$_2$, much earlier than was seen in intact rats. At 4% and 6% CO$_2$, $\text{ABS}_m$ activity started at the end of the breathing cycle (late $E2$), and terminated early in the following inspiration. At 6% CO$_2$, $T_{E2}$ was significantly prolonged (Figure 2.4).
2.3.2 Hypoxia

In unanesthetized animals, progressive hypoxia led to a significant increase in breathing frequency ($p<0.001$) accompanied by an insignificant decrease in tidal volume (Figure 2.5A, B). The hypoxia induced increases in breathing frequency were due to a significant decrease in the inspiratory interval ($T_I$) ($p=0.026$) and the phase of expiratory braking ($T_{E1}$) ($p<0.001$). There was also a trend for the expiratory pause ($T_{E2}$) to decrease but this never reached significance (Figure 2.6A-C). Breathing was exclusively diaphragmatic and active expiration was never recruited (Figure 2.7). There was no significant change in the total amount of the diaphragm muscle activity associated with each breath.

Following anesthesia there was a trend for breathing frequency to increase in normoxic normocapnia but this trend never achieved significance (Figure 2.5C, D). The hypoxic ventilatory response was similar to that seen in unanesthetized animals (an increase in breathing frequency) but now not significant. There were no significant changes in $T_I$ (Figure 2.6A). The increase in breathing frequency was due to non-significant reductions in $T_{E1}$ and $T_{E2}$ (Figure 2.6E, F). Now, however, breathing was not exclusively diaphragmatic; the $INT_m$, were more active in normoxic normocapnia, and their activity contributed to inspiration ($T_I$) and expiratory braking ($T_{E1}$) (Figure 2.7).

2.4 Discussion

2.4.1 Caveats

The purpose of this study was 1) to determine when and how active expiration (AE) is recruited into the breathing cycle, 2) to determine whether recruitment of $INT_m$ and $ABS_m$
activity within the breathing cycle is fixed or flexible and 3) to determine whether the presence and timing of this activity is influenced by vagal feedback and anesthetics. Before addressing each of these questions, however, we must point out three important caveats that influence the interpretation of our data.

The first caveat is that our interpretation of the timing of all events is based on a combination of impedance measurements of chest wall movement and DIA$_m$ activity. We set the start of inspiration (I) as the start of chest wall expansion and DIA$_m$ activity and the start of expiration (E1) as the start of chest wall compression. We set the end of E1 and the beginning of E2 as the point at which DIA$_m$ activity ceases. The end of E2 will be the beginning of I. We did not measure airflow in this study and the precise timing of the phases of inspiratory and expiratory airflow may not correspond perfectly with changes in chest wall movements. Any differences, however, will be small and should not alter the overall conclusions of the study. Also, note that the expiratory braking activity of upper airway muscles may still continue after the DIA$_m$ E1 activity ceases. Thus our phasing is based exclusively on the activity of pump muscles.

The second caveat concerns our interpretation of INT$_m$ activity. Placement of the INT$_m$ EMGs was within the lateral T11-T13 costal space. Given how thin the intercostal muscles are, the INT$_m$ EMG electrodes would pass through both the external and internal intercostal muscles. Most of the INT$_m$ EMG activity we recorded was present during inspiration and we assume that it reflected the activity from the external intercostal muscles. When this activity carried over into E1 we assume that it was still external intercostal activity contributing to expiratory braking. When activity was present during E2, however, the INT$_m$ activity appeared biphasic (see Figure 2.8) and the activity occurring in E2 most likely represented activity from the internal intercostal
muscles. We would also point out that INT\(_m\) associated with T11-T13 would be the last of the INT\(_m\) to be recruited. It is well established that there is a progressive rostrocaudal and dorsoventral recruitment of individual intercostal muscles (dogs: De Troyer and Legrand, 1995; cats: Greer and Martin, 1990; humans: De Troyer et al., 2003; De Troyer et al., 2005). A similar rostrocaudal recruitment is found with the neural output recorded from newborn rats using the \textit{in vitro} brainstem-spinal cord preparation (Giraudin et al., 2008; Chapter 4. It is conceivable therefore that recruitment of more rostral INT\(_m\) could have occurred earlier than what is reported here for the more caudal muscles.

The third caveat also concerns our interpretation of the INT\(_m\) activity, as the progressive recruitment of this activity might also reflect crosstalk from increased DIA\(_m\) activity coincident with an increase in \(V_T\). However, while unanesthetized rats exhibited a significant increase in tidal volume, this was not accompanied by a significant increase in diaphragm EMG activity. Furthermore, in anesthetized rats, there was a recruitment of the INT\(_m\) without a substantial increase in tidal volume or a significant increase in diaphragm activity. The activity patterns of the INT\(_m\) in anesthetized and unanesthetized animals were also different (showing altered changes in duration and coordination with increased hypercapnia) that could not arise as an artifact of enhanced DIA\(_m\) activity. Taken together these observations support our interpretation that INT\(_m\) activity was due to recruitment of the INT\(_m\) and not due to crosstalk from increased DIA\(_m\) activity.

### 2.4.2 Recruitment of the abdominal muscles

In the present study rats did not recruit intercostal muscles within the lower third of the ribcage (T10-T13), or their abdominal muscles, under normoxic normocapnic conditions. Thus
contraction of the diaphragm and passive expiration were sufficient to power the movement of air, producing the typical three phase breathing cycle (I, E1, E2). This is consistent with previous studies (Richter, 1982; 1996) and typical of almost all mammals (see Richter and Smith, 2014 for review) with very few exceptions being noted to date (horses: Koterba et al., 1988, dogs: De Troyer et al., 1989, opossums: Reilly and White, 2009).

Hypercapnia was the only experimental treatment that recruited the expiratory abdominal muscles. In rats with intact vagi, active expiration first appeared at 8% inspired CO₂ and persisted as CO₂ levels increased to 10%. This is consistent with the results of Abdala et al., (2009) who found, using an in situ preparation, that abdominal nerve activity first appeared intermittently at moderate levels of CO₂ (7 % CO₂ balanced with air) and that when the preparation was perfused with 10% CO₂, abdominal nerve activity was present with every fictive breath (Abdala et al., 2009). The 8% level of CO₂ used in our study was sufficient to recruit expiratory abdominal muscle activity with every breath.

The ABSₘ activity appeared either during late E2, or throughout the entirety of the expiratory phase. This is also consistent with the majority of studies that have produced active expiration in mammals with increasing respiratory drive (humans: Abe et al., 1996; rats: Sherrey et al., 1988; Janczewski et al., 2002; Abdala et al., 2009; Taccola et al., 2007; Pagliardini et al., 2011; cats: Fregosi et al., 1987; dogs: Ledlie et al., 1983; De Troyer et al., 1989; and goats: O’Halloran et al., 1999). It has been hypothesized that this timing within the breathing cycle, coming at the end of the ventilatory pause with the lungs at functional residual capacity, correlates with the recruitment of the expiratory reserve volume (ERV) (Pagliardini et al., 2011; Jenkin and Milsom, 2014). In the present study, active expiration was associated with significant increases in tidal volume (400 to 600%). Taken together, the data suggest that the initial increase
in tidal volume in animals breathing low levels of CO\textsubscript{2} is due to recruitment from the inspiratory reserve volume (IRV) and that this is subsequently complimented by recruitment from the ERV via active expiration at higher levels of inspired CO\textsubscript{2}.

In most cases, the ABS\textsubscript{m} activity extended into the following I phase. As this only occurred when animals were producing large tidal volumes, this activity might be generating an inspiratory braking phase. This potential resistance to diaphragmatic activity may assist initial thoracic expansion reducing paradoxical chest wall movement, and help further recruit the IRV.

It has been shown that ABS\textsubscript{m} activation arises from an expiratory rhythm generator that resides in the retrotrapezoid nucleus/parafacial respiratory group (RTN/pFRG). Pagliardini et al., (2011) found that injection of bicuculine/strychnine into this area in adult Sprague-Dawley rats allowed the expiratory rhythm generator to become active under normoxic, normocapnic conditions. This suggests that recruitment of late expiratory abdominal nerve activity is due to disinhibition of the RTN/pFRG by increased drive.

No expiratory abdominal muscle activity was observed under hypoxic conditions, despite a 40-50% increase in breathing frequency. Fregosi et al., (1987) found that hypoxia inhibited phasic abdominal expiratory nerve (L1 nerve) activity in decerebrate cats and speculated that the inhibition may be due to a respiratory alkalosis associated with the increase in breathing frequency reducing the P\textsubscript{CO2} and [H\textsuperscript{+}] in the medulla. Similarly, Smith et al., (1989) found that when dogs were exposed to hypoxia, inspiratory muscle EMG activity increased, but expiratory muscle activity either decreased or remained unchanged. Along with the decrease in abdominal activity, Fregosi \textit{et al.}, (1987) proposed an increase in the functional residual capacity (FRC) that would occur with a decrease in expiratory abdominal activity in addition to a known hypoxia-induced increase in expiratory braking (Bartlett, 1980; England et al., 1982). This suggests that
the expiratory interval is insufficient for complete passive exhalation, in part due to the shorter
expiratory interval and in part due to the increased airway resistance that occurs due to hypoxia-
induced glottal narrowing. An elevated FRC would likely increase elastic recoil that in turn
would counteract these expiratory braking effects. If present under these increased expiratory
braking conditions, the expiratory abdominal muscles would be detrimental, increasing pressure
on the airways causing them to narrow further. This suggests there may be mechanisms in place
to actively inhibit the recruitment of $\text{ABS}_m$ under these conditions and suggests that increasing
drive to increase breathing frequency versus tidal volume produce different effects.

$\text{ABS}_m$ activity was also inhibited by urethane anesthesia. Rats anesthetized with
isoflurane (Iizuka, 2009) showed minimal phasic abdominal EMG activity compared to
unanesthetized rats and dogs anesthetized with halothane (Leevers and Road, 1995) had less
phasic expiratory abdominal muscle contractions than unanesthetized dogs. However, there is
also evidence that anesthetics enhance phasic expiratory muscle activity. Humans anesthetized
with halothane (Warner et al., 1995), dogs anesthetized with pentobarbital (Warner et al., 1992)
and some cats (4 of 7) anesthetized with ketamine (Fregosi et al., 1987), exhibited an increase in
phasic abdominal muscle activity. Interestingly, it is the anesthetics that often depress tidal
volume and increase breathing frequency, such as urethane (Field et al., 1993) and isoflurane
(Brunton et al., 2005), which tend to inhibit expiratory abdominal muscle recruitment.

Vagotomy led to a large increase in tidal volume and reduction in breathing frequency as
expected (Mörschel and Dutschmann, 2009). With progressive hypercapnia, rats increased
ventilation with further increases in tidal volume and did not modify their breathing frequency.
AE was recruited when these increases in $V_T$ became significant. Following vagotomy, $\text{ABS}_m$
activity was recruited at lower levels of respiratory drive; the rats now recruited their expiratory
abdominal muscles at around 5% CO$_2$ (from 4% CO$_2$ to 6% CO$_2$, depending on the animal). This suggests that vagal feedback inhibits active expiration. Lemes and Zoccal (2014) also found this to be the case in anesthetized, tracheostomized rats. Breathing 7 and 10% inspired CO$_2$ produced active expiration post-vagotomy but not pre-vagotomy. Furthermore, post-vagotomy breathing was slow and tidal volume was large and now severe hypoxia (7% O$_2$) only produced further increases in tidal volume, rather than in breathing frequency, that were also associated with active expiration. Similarly, Iizuka and Fregosi (2007) found in vagotomized, paralyzed and ventilated rats, both hyperoxic hypercapnia (9-12% CO$_2$) and isocapnic hypoxia (10% O$_2$) produced substantial late-E activity in the iliohypogastric nerve reflecting abdominal muscle activity.

The role of afferent vagal feedback on the recruitment of phasic expiratory abdominal activity, however, is complex. It has been shown to be influenced by the extent and rate at which the lungs are inflated and deflated (changing phasic or tonic feedback), by species differences, and to depend on which abdominal muscle is studied. Bishop found that expiratory activity recorded from the external oblique muscles became progressively more active with positive pressure breathing (1962). They found that during positive pressure breathing (enhancing tonic vagal feedback), the work of moving air transitioned from an inspiratory act – recruiting the inspiratory reserve volume - to an expiratory act – recruiting the expiratory reserve volume. This transition from a greater inspiration to a forced expiration was dependent on afferent vagal feedback from the pulmonary stretch receptors, as after vagotomy this response was abolished (Bishop, 1962; Bishop and Bachofen, 1973). Surprisingly, Leevers and Road found that dogs exhibited an increase in transversus abdominis muscle activity during expiratory threshold loading (enhancing tonic activity) that was not modified by vagotomy, suggesting that the
abdominal muscles are also under extra-vagal control (1995). Consistent with this, with negative pressure breathing (reducing tonic feedback), expiratory abdominal muscle activity was completely silenced. During negative pressure breathing, lung volumes are low, resulting in a decrease in the expiratory reserve volume (Bishop, 1962). With no expiratory reserve volume to draw on, there may be no benefit of recruiting the expiratory abdominal muscles. There was also an increase in expiratory abdominal activity when withholding lung inflation in cats (removing phasic vagal feedback; Fregosi et al., 1990) and this response was not modified by vagotomy. Whether the discrepancy in the results is due to species differences, or the recording of different abdominal muscles, remains unclear. Based on the results outlined above, however, under eupneic conditions, phasic vagal feedback inhibits AE unless tonic activity is enhanced (results outline in Table 2.1).

Vagotomy also altered the timing of \( \text{ABS}_m \) activity. While both vagi intact and vagotomized unanesthetized rats exhibited \( \text{ABS}_m \) activity at the end of the expiratory phase (producing a four-phase breathing cycle of: I, E1, E2, AE), because \( T_{E2} \) was so prolonged post-vagotomy, AE occupied a smaller portion of the expiratory cycle when compared with vagi intact rats. The active expiration still occurred immediately before inspiration (during E2) and terminated at the beginning of the following inspiration (4% CO\(_2\) and 6% CO\(_2\); Figure 2.8). This was not the case in the study of Lemes and Zoccal (2014) where abdominal muscle activity was present throughout E2, but their animals were both anesthetized and tracheostomized.

The sum of these data present an interesting paradox; active expiration appears to be recruited to produce a larger tidal volume, while a large tidal volume increases vagal feedback from pulmonary stretch receptors (PSR) that in turn inhibits active expiration. This suggests that the increase in disinhibition from increasing drive must exceed the increasing inhibition from
PSR feedback. Note that removal of the inhibition from the PSR alone (vagotomy) was insufficient to produce active expiration. Despite the large increase in tidal volume and end-inspiratory lung volume post-vagotomy, there was no active expiration under normoxic normocapnic conditions. Adding a little CO₂, however, elicited active expiration. Current models of the neural circuitry that powers breathing suggest that disinhibition may arise from excitation of chemosensitive cells in the RTN (Guyenet et al., 2009; Molkov et al., 2014), highlighting the importance of CO₂ sensitivity and a drive to increase tidal volume in generating active expiration.

The initial increase in Vₜ without AE suggests that the IRV is recruited before AE recruits the ERV. This suggests that while initial increases in tidal volume are due exclusively to recruiting from the IRV, as end-tidal lung volumes become large, active expiration is recruited to produce further increases in tidal volume without further lung expansion. Thus, another scenario is that as PSR feedback increases, it not only inhibits further inspiration, it excites active expiration recruiting further increases in tidal volume without producing further increases in end-inspiratory lung volume. PSR and chest wall feedback act to restrict recruitment of the IRV and promote recruitment of the ERV. The chest wall afferents are not as effective in this role and thus removing PSR feedback by vagotomy results in a larger tidal volume before the chest wall afferents activate AE. In this scenario, drive only affects active expiration indirectly by its effects on lung volume and PSR and chest wall afferent feedback.

2.4.3 Recruitment of the intercostal muscles

In unanesthetized rats, external INTₘ activity was recruited as Vₜ began to increase in hypercapnia. The inspiratory interval, Tᵢ, did not change suggesting that the INTₘ were recruited
to assist in producing higher levels of inspiratory air flow than could be produced by the diaphragm alone. Activity in this muscle group continued into T₁ suggesting that the INTₘ also assist with expiratory braking. Surprisingly, the INTₘ were not recruited as breathing frequency increased in hypoxia, but tidal volume was reduced under these conditions suggesting that despite the reduction in T₁, airflow velocity may not have increased.

Interestingly, when rats were anesthetized in our study, phasic bursts of external intercostal muscle activity appeared both under normoxic normocapnic conditions as well as throughout the hypoxic and hypercapnic trials. This suggests that INTₘ activity is inhibited under eupneic conditions. Similar observations have been made in dogs (Bouvertot and Fitzgerald, 1962), cats (Sears et al., 1982) and kittens (Guthrie et al., 1990), where there was an increase in external intercostal (inspiratory muscle) activity when animals were anesthetized. While the reason for this inhibition of intercostal muscle activity in eupnea is unclear, it appears that expression of INTₘ activity, just as with ABSₘ activity, requires disinhibition. In both cases, this appears to require increased drive leading to either increased tidal volume or inspiratory airflow, with the threshold for disinhibition of the INTₘ being lower than that for the ABSₘ.

As mentioned earlier, given the placement of the INTₘ EMG electrodes in the present study, (the lateral T11-T13 costal space) and how thin the intercostal muscles are, we were unable to clearly distinguish between recruitment of the internal versus the external INTₘ other than by deduction based on with what chest wall movements the activity correlated. Using this logic, however, there were two instances where we felt internal INTₘ activity was present, both in vagotomized animals at the high levels of hypercapnia where AE was also present. In Figure 2.8, immediately preceding inspiration there is an inward movement of the chest wall in this vagotomized animal while breathing 2 to 4% CO₂ (red arrows). This was accompanied by a
small burst of activity in the INT\textsubscript{m} as well as activity in the ABS\textsubscript{m}. The activity in the INT\textsubscript{m} slightly preceded that in the ABS\textsubscript{m} consistent with a rostrocaudal spread of motor recruitment. This suggests that the internal intercostal muscles are recruited along with the abdominal muscles to produce active expiration. We did not see this in animals with their vagi intact.

Half of the vagotomized rats studied also expired to levels below the functional residual capacity at the end of the E1 phase (black arrows; Figure 2.8). There was no ABS\textsubscript{m} activity during the E1 phase suggesting that this expiration was due to the activity of the internal intercostal muscles. Internal intercostal muscles may be recruited at this time to overcome the laryngeal paralysis that is often observed in vagotomized rats and may act to counter the reduced or restricted airflow during this part of the breathing cycle (Anrep and Samaan, 1932; Sharpey-Schafer, 1932; Kamosińska and Szereda-Przestaszewska, 1988).

### 2.4.4 Control of the phases of the breathing cycle

T\textsubscript{1} is tightly regulated. It varies the least of all phases. Under hypercapnic conditions (both anesthetized and unanesthetized with the vagi intact or after bilateral vagotomy) there was never any change to T\textsubscript{1}. Under hypoxic conditions in unanesthetized rats, there was a decrease in T\textsubscript{1} at the highest level of hypoxia (9% O\textsubscript{2}). It would appear that increasing tidal volume with progressive hypercapnia is due to increasing airflow velocity rather than lengthening T\textsubscript{1}. Increasing breathing frequency with progressive hypoxia, on the other hand, was due to shortening T\textsubscript{E1} and T\textsubscript{E2} rather than shortening T\textsubscript{1}.

The majority of the change in the breathing cycle with increasing breathing frequency was due to a significant decrease in T\textsubscript{E1}. Since E1 is characterized by post-inspiratory activity, expiratory braking is reduced. Based on the importance of the pons in generating and regulating
post-inspiratory or E1 inspiratory motor activity (Mörschel and Dutschmann, 2009; Smith et al., 2009), it seems likely that the pons is also critical in modulating the changes in $T_{E1}$ observed here.

Anesthesia also had little effect on $T_I$. Increases in breathing frequency were reduced by anesthesia but the overall trends were similar. Anesthesia and vagotomy eliminated the shortening of $T_{E1}$ with increasing CO2 but they also eliminate any increases in breathing frequency.

$T_{E2}$ was very plastic. With progressive hypercapnia, it was reduced as breathing frequency increased but once $V_T$ started to increase significantly on high levels of CO2, it was prolonged. This occurred even when active expiration was not involved (as in anesthetized animals), but especially when it was.

### 2.4.5 General conclusions

The present study confirms that in rats, active expiration is recruited at the end of the expiratory pause (late E2) and serves to expand tidal volume drawing from the expiratory reserve volume. It also confirms that vagal afferent input, most likely from pulmonary stretch receptors, inhibits the emergence of active expiration in hypercapnia. It demonstrates that removal of this inhibition alone is insufficient to produce active expiration without an increase in respiratory drive leading to increased airflow and tidal volume. This suggests that in intact animals an increase in disinhibition from increasing respiratory drive must exceed the increasing inhibition from PSR feedback to recruit active expiration as tidal volume increases. Our data further suggest that external intercostal muscle activity is also inhibited under eupneic conditions. While the reason for this inhibition of intercostal muscle activity in eupnea is unclear, it appears that
expression of intercostal muscle activity to assist inspiration also requires disinhibition. The threshold for disinhibition of the intercostal muscles is lower than that for the abdominal muscles. When abdominal muscle activity was recruited at the end of the expiratory pause, while diaphragmatic activity still occurred in three phases (I, E1, E2), chest wall movement and airflow consisted of four phases; I, E1, E2, and AE (or E3).
Breathing
∫DIA
raw DIA
∫INT
Raw INT
∫ABSm
Raw ABSm
A. B. C.
I E1 E2
INTm
ABSm
Figure 2.1 Representative trace of the durations and offsets of different muscles. A) Vertical dotted lines identify the measurements of the I phase, E1 phase and E2 phase of the diaphragm (DIA<sub>m</sub>). B) The vertical dotted lines identify the start and end of the intercostal muscle activity (INT<sub>m</sub>). C) The vertical dotted lines identify the start and end of the abdominal muscle activity (ABS<sub>m</sub>).
Unanesthetized

A.

B.  

Anesthetized

C.  

D.  

Vagotomized

E.  

F.  

Note: The figures depict the effects of different CO₂ concentrations on tidal volume and breathing frequency across unanesthetized, anesthetized, and vagotomized conditions.
Figure 2.2 Effect of progressive hypercapnia on the tidal volume (measured with impedance; V) and breathing frequency (min⁻¹) of unanesthetized (A, B) and anesthetized rats (C, D) with vagi intact, and for vagotomized rats (E, F). Significant differences within progressive hypercapnia are represented with letters (a,b,c) and significant differences between vagi intact unanesthetized rats (*) and vagi intact anesthetized rats (#) under eupneic conditions (0% CO₂) are presented with symbols, $p<0.05$. 
Figure 2.3 Effect of progressive hypercapnia on the duration of the inspiratory, expiratory braking and second expiratory phases. Values presented for the duration of the inspiratory ($T_I$; sec), expiratory braking ($T_{E1}$; sec) and second expiratory phase ($T_{E2}$; sec) of unanesthetized (A, B, C) and anesthetized rats (D, E, F) with vagi intact, and for vagotomized rats (G, H, I). Significant differences within progressive hypoxia are represented with letters (a,b), $p<0.05$. 
Figure 2.4 Effect of progressive hypercapnia on the duration and the coordination of the respiratory muscles of unanesthetized, anesthetized and unanesthetized vagotomized rats. Values presented for the duration of the diaphragm (DIA<sub>m</sub>) muscle’s inspiratory (I, blue bars), expiratory braking (E1; green bars) and second expiratory phase (E2; yellow bars) activity. Values are also presented for the duration and coordination of the internal intercostal (INT<sub>m</sub>) muscle activity (purple bars) and the abdominal (ABS<sub>m</sub>) muscle activity (red bars). Significant differences within progressive hypoxia are represented with letters (a,b,c), p<0.05.
Figure 2.5 Effect of progressive hypoxia on the tidal volume (measured with impedance; V) and breathing frequency (min⁻¹) of unanesthetized (A, B) and anesthetized rats (C, D). Significant differences within progressive hypoxia are represented with letters (a,b), p<0.05.
Figure 2.6 Effect of progressive hypoxia on the duration of the inspiratory, expiratory braking and second expiratory phases. Values presented for the duration of the inspiratory (TI; sec), expiratory braking (TE1; sec) and second expiratory phase (E2; sec) of unanesthetized (A, B, C) and anesthetized rats (D, E, F). Significant differences within progressive hypoxia are represented with letters (a,b), $p<0.05$. 
Figure 2.7 Effect of progressive hypoxia on the duration and the coordination of the respiratory muscles. Values presented for the duration and the coordination of the diaphragm (DIA$_m$) muscle’s inspiratory (I, blue bars), expiratory braking (E1; green bars) and second expiratory phase (E2; yellow bars) activity. Also showing the internal intercostal (INT$_m$) muscle activity (purple bars) for unanesthetized and anesthetized rats. Significant differences across progressive hypoxia are represented with letters (a,b), $p<0.05$. 
Figure 2.8 Representative traces of the respiratory muscle recruitment of bilaterally vagotomized rat across progressive hypercapnia. Traces of one breath (measured with impedance, upward slope represents inspiration), the raw and integrated diaphragm (DIA<sub>m</sub>), intercostal muscle (INT<sub>m</sub>) and abdominal muscle (ABS<sub>m</sub>) activity of a bilaterally vagotomized rat at 0%, 2%, 4% and 6% inspired CO<sub>2</sub>. Red arrows represent a late-expiratory active expiration; black arrows represent an early-expiratory active expiration.
Table 2.1 The impact of tonic and phasic vagal feedback on the recruitment of active expiration. Results are based on a number of studies as outlined in the text.

<table>
<thead>
<tr>
<th>Study</th>
<th>Modification to Vagal feedback</th>
<th>Tonic</th>
<th>Phasic</th>
<th>AE response</th>
<th>Effect of vagotomy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Everyone</td>
<td>Control</td>
<td>Present</td>
<td>Present</td>
<td>No AE</td>
<td></td>
</tr>
<tr>
<td>Bishop, 1962</td>
<td>Positive pressure</td>
<td>Increased</td>
<td>Present</td>
<td>AE</td>
<td>Abolished by vagotomy</td>
</tr>
<tr>
<td>Leivers and Road, 1995</td>
<td>Expiratory loading</td>
<td>Increased</td>
<td>Present</td>
<td>AE</td>
<td>Not modified by vagotomy</td>
</tr>
<tr>
<td>Bishop, 1962</td>
<td>Negative pressure</td>
<td>Reduced</td>
<td>Present</td>
<td>No AE</td>
<td></td>
</tr>
<tr>
<td>Fregosi et al., 1990</td>
<td>Withholding a breath</td>
<td>Present</td>
<td>Removed</td>
<td>AE</td>
<td>Not modified by vagotomy</td>
</tr>
<tr>
<td>Chapter 2</td>
<td>Vagotomy</td>
<td>Removed</td>
<td>Removed</td>
<td>AE</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 3: The Kölliker-Fuse Nucleus acts as a timekeeper for late-expiratory abdominal activity

3.1 Introduction

Breathing is dependent on multiple motor outputs whose activities are precisely timed to the transition between the phases of the breathing cycle. At rest, the mammalian breathing cycle is composed of three phases (Richter, 1982; Smith et al., 2007). The first phase, inspiration (I), is dependent on the active contraction of the inspiratory muscles to draw air into the lungs. During the second phase (E1), there is prolonged inspiratory muscle activity (primarily the diaphragm) and laryngeal muscle contraction counteract the elastic recoil forces of the lungs and chest wall to control the rate that air is exhaled from the lungs (Davis and Bureau, 1987; Gautier et al., 1973; Dutschmann et al., 2014; Feldman et al., 2013). During the third phase (E2), there is no muscle contraction and no airflow (Richter, 1996; Feldman and McElrion, 2003). Thus, under eupneic conditions, expiration is considered to be passive (Jenkin and Milsom, 2014).

The three-phase eupneic breathing pattern is the product of the coordinated activities of the respiratory central pattern generator (CPG) located in the brainstem, especially within the ventral respiratory column (VRC) of the medulla (Smith et al., 2007). The current hypothesis is that inspiratory neurons of the preBötzing Complex (preBötC) generate inspiratory activity, while neurons of the Bötzing Complex (BötC), essentially post-inspiratory (post-I) and augmenting expiratory (aug-E) neurons, control the expiratory phase (Smith et al., 1991; Richter, 1996; Onimaru and Homma, 2003; Janczewski and Feldman, 2006; Alheid and McElrion, 2008; Smith et al., 2009, 2013). The respiratory rhythm generator circuitry is certainly complex, involving redundant and degenerate regions that contribute to respiratory pattern formation.
(Jones and Dutschmann, 2016). At its simplest level, however, neurons from the preBötC and BötC establish mutual connections with pontine and medullary respiratory groups, sending projections to cranial and spinal pre-motor and motor nuclei to coordinate the muscle contraction and relaxation cycles that underpin breathing under resting conditions (Smith et al., 2013).

Under conditions of elevated respiratory drive (i.e. hypercapnic or hypoxic conditions), expiration often becomes an active process, and recruitment of the abdominal muscles forcefully exhales air from the lungs (Lemes and Zoccal, 2014; Jenkin and Milsom, 2014). The emergence of active expiration relies on the activation of a conditional active expiratory oscillator located in the ventral medulla, rostral to the VRC, within the retrotrapezoid nucleus/parafacial respiratory group (RTN/pFRG) (Janczewski and Feldman, 2006). It has been hypothesized that the RTN/pFRG provides excitatory input to pre-motor expiratory neurons of the VRC, critical for powering the forceful contraction of the abdominal muscles during the late part of the expiratory phase (late-E) (Molkov et al., 2010; Pagliardini et al., 2011, Silva et al., 2016). However, the contribution of other brainstem respiratory areas in the generation and coordination of active expiration remains unclear.

Excitatory drive from the pons is necessary for generating abdominal muscle burst activity during the late-E phase (Abdala et al., 2009; Molkov et al., 2010). Within the dorsal pons, there is a group of respiratory neurons of the Kölliker-Fuse nucleus (KF), which interact with and modify the output of the medullary areas described above and is critical for the transition from inspiration to expiration (Dutschmann and Herbert, 2006). The KF is suggested to send excitatory inputs to the VRC neurons, mainly to BötC post-I neurons, which are essential for terminating inspiration and regulating post-I activity in the phrenic and cervical vagus nerves (Smith et al., 2007). The KF also provides the drive that excites upper airway musculature
contraction during the early (post-I) phase of expiration (Dutschmann and Herbert 2006). While insight into the role of the KF and the dorsolateral pons in controlling the transition between inspiration and expiration is accumulating, little attention has been paid to the role of the pons in regulating the transition from the post-I to the late-E phase, or in generating or modifying the transition from a eupneic passive to an active expiratory pattern when drive is elevated. Therefore, we hypothesized that the KF also controls the timing of the phase transition from the post-I to the late-E phase, as well as the onset of late-E activity in abdominal motor nerve output. Understanding the role the pons (specifically the KF) in the neural circuitry responsible for the generation of active expiration under both hypercapnic and hypoxic conditions is critical to our understanding of a number of respiratory disorders that effect expiration (such as chronic obstructive pulmonary disease (COPD)), in addition to furthering our understanding of the neuromuscular control of breathing.

3.2 Methods

The experimental procedures followed the guidelines of the Brazilian National Council for Animal Experimentation Control (CONCEA) and of the US National Institutes of Health (NIH, publication no. 85-23, 1996). All experimental protocols were authorized by the Local Ethical Committee in Animal Experimentation of the School of Dentistry of Araraquara (protocol 18/2014) and the University of British Columbia (protocol A-13-0025).
3.2.1 Experimental animals

Juvenile male Holtzman rats (60-80 g) were used in the present study. Animals were housed under controlled conditions of temperature (22 ±1 °C) and humidity (50-60%) under a 12-h light/dark cycle (lights on at 07:00 am) with free access to rat chow and water.

3.2.2 The in situ working heart brain preparation

Working heart-brainstem preparations (Paton, 1996) were surgically prepared, as previously described (Zoccal et al., 2008). Juvenile Holtzman rats were deeply anaesthetized with halothane until the paw and tail pinch reflexes were abolished. The animals were then transected below the diaphragm and submerged in a cold Ringer solution (in mM: NaCl, 125; NaHCO₃, 24; KCl, 3; CaCl₂, 2.5; MgSO₄, 1.25; KH₂PO₄, 1.25; dextrose, 10). Animals were then decerebrated, and their skin was removed. The lungs were removed with care to not damage the descending aorta or the phrenic nerve at the attachment to the diaphragm. The left cervical vagus nerve was isolated. Preparations were then transferred to a recording chamber; the descending aorta was cannulated and perfused retrogradely (21-25 mL.min⁻¹; Watson-Marlow 502s, Falmouth, Cornwall, UK), via a double-lumen cannula, with Ringer solution containing 1.25% Polyethylene glycol (an oncotic agent, Sigma, St Louis, USA) and vecuronium bromide (a neuromuscular blocker, 3-4 µg.mL⁻¹). The perfusion pressure was held within 50–70 mmHg by adding vasopressin (0.6 – 1.2 nM, Sigma, St. Louis, MO, USA) to the perfusate. The perfusate was continuously gassed with 5% CO₂ and 95% O₂, warmed to 31–32°C and filtered using a nylon mesh (25 µm).
3.2.3 Data acquisition and analysis

Respiratory motor nerves were isolated and recorded using glass suction bipolar electrodes held in place with micromanipulators. The left phrenic nerve (PN) discharge was recorded from its central end. The left vagus nerve (cVN) was cut at the cervical level (below the carotid artery bifurcation) and its efferent activity was recorded. An abdominal nerve (AbN) was isolated from the abdominal muscles on the right at thoracic–lumbar level (T12–L1), cut distally and its activity recorded. All the signals were amplified, band-pass filtered (0.3–3 kHz) and acquired in an A/D converter to a computer using Spike 2 software (Cambridge Electronic Design, CED, Cambridge, UK). The analyses of the activities of the phrenic, cervical vagus, and abdominal nerves were carried out on the rectified and smoothed signals (time constant of 50 msec) and performed off-line using Spike 2 software with custom-written scripts (as described by Zoccal et al., 2008). PN activity was analysed by its burst amplitude (µV), frequency (cycles per minute, cpm), duration (time of inspiration, s) and interval (time of expiration, s). For the cVN, inspiratory (coincident with PN burst) and post-inspiratory (remaining activity during the expiratory period) components were measured as mean activity (µV). The cVN I and post-I activity and AbN late-E activity was also analyzed by their amplitude (µV) and duration (s). The respiratory responses elicited by hypercapnia and KCN were evaluated as the maximal variation and the changes were expressed in their original units or in percentage variation in relation to respective baseline values.

3.2.4 Activation of peripheral and central chemoreceptors

The in situ preparations were exposed to potassium cyanide (KCN) (N=13) or hypercapnia (N=10) to activate peripheral and central chemoreceptors and generate active
expiration. Exposure was randomized for each trial. The peripheral chemoreceptors were stimulated through injections of KCN (50 µL, 0.05%) into the descending aorta via the perfusion cannula. The central chemoreceptors were stimulated with hypercapnia (N=10) by raising the fractional concentration of CO₂ in the perfusate from 5 to 8-10% (balanced with O₂) for 5-10 min, using a gas-mixing device (AVS Projetos, São Carlos, Brazil) connected to cylinders of pure O₂ and CO₂. Both stimuli produced consistent respiratory reflex responses, with low variability among preparations.

3.2.5 Microinjections into the Kölliker-Fuse

Bilateral microinjections of isoguvacine (a GABAₐ agonist, 10 mM, Sigma-Aldrich) containing 5% fluorescent latex microbeads (Lumafluor, New City, NY, USA) were performed into the KF to pharmacologically inhibit this region. Before the isoguvacine microinjections, the KF was functionally identified by microinjections of L-glutamate (10 mM, Sigma-Aldrich), which evoked an increase in cVN post-I activity and PN apnea (Bautista and Dutschmann, 2014), as demonstrated in Figure 3.1. The glass pipettes contained either the drug-microbead mixture or L-glutamate and were positioned in the KF using the following stereotaxic coordinates: 5.5 mm rostral from the calamus scriptorius; 1.75 mm lateral from midline; and 1.5 mm ventral to the dorsal surface. The volume of each microinjection was 70-90 nL. L-glutamate microinjections were performed at the beginning of the experimental protocol. Microinjections of isoguvacine were performed either during the hypercapnic stimulus or 20-60 seconds before the injections of KCN. Bilateral microinjections were achieved in less than 2 min and the effects were observed for 1-5 min after injections.
3.2.6 Histology

At the end of each experiment, the brainstem was removed and fixed by immersion for 5 days in 10% buffered formalin and then in sucrose solution (20%) overnight. After, 40 μm coronal sections were obtained at the level of KF (Leica, CM1850 UV; Wetzlar, Hesse, Germany) and analysed in a fluorescent microscope (Leica, DM5500 B) with the aid of a rat brain Atlas (Paxinos and Watson, 2009) to identify the injections sites (fluorescent microbeads; Figure 3.2).

3.2.7 Statistical analysis

All results are expressed at mean ± standard errors of the means, and comparison between conditions was done with repeated measures ANOVA. If normality or equal variance was not passed, the statistical test was performed on ranks. Holm-Sidak and Tukey post-hoc tests were used as appropriate. The comparisons were conducted with Sigma Stat 11 software (Dundas Software LTD) and differences were considered significant when \( p < 0.05 \).

3.3 Results

Under basal conditions (95% \( \text{O}_2 \) and 5% \( \text{CO}_2 \)), all in situ preparations exhibited a eupneic-like breathing pattern, with ramping PN bursts, biphasic cVN activity consisting of an inspiratory component followed by decrementing post-I activity, and tonic low-amplitude AbN expiratory activity (Figure 3.3A).
3.3.1 Effect of isoguvacine microinjections in the KF on baseline activities

Figure 3.3A shows a representative trace demonstrating the effect of inhibiting the KF with bilateral injections of isoguvacine on the respiratory related neural output. After bilateral inhibition of the KF with isoguvacine during baseline conditions there was a significant decrease in PN amplitude (12.94 ± 0.74 vs. 10.12 ± 0.76 µV, \( p=0.003 \), Figure 3.3C), but not in PN frequency (14.63 ± 1.84 vs. 11.65 ± 3.26 bpm, Figure 3.3B). Moreover, isoguvacine microinjections in the KF significantly increased inspiratory time (1.09 ± 0.07 vs. 1.81 ± 0.25 sec, \( p=0.001 \), Figure 3.3D), but had no effect on the expiratory time (3.48 ± 0.58 vs. 3.94 ± 0.81 sec, \( p=1.000 \), Figure 3.3E). Bilateral inhibition of the KF significantly increased cVN inspiratory area under the curve (AUC; 12.94 ± 0.74 vs. 10.12 ± 0.76 µV, \( p=0.001 \); Figure 3.3F) and significantly decreased cVN post-I AUC (13.73±1.30 vs. 18.64±2.73 µV, \( p<0.001 \); Figure 3.3G) Bilateral inhibition of the KF had no significant effect on AbN mean activity during E1 (2.42 ± 0.74 vs. 2.34±1.14 µV, Figure 3.3H) or during E2 (4.13±0.82 vs. 4.49 ± 1.30 µV, Figure 3.3I).

3.3.2 Effect of KF inhibition on the peripheral O₂ chemoreflex activation

Stimulation of the peripheral chemoreceptors with KCN (N=13, Figure 3.4A, B) elicited a transient tachypneic response (14.39 ± 1.01 vs. 22.72 ± 1.42 bpm, \( p<0.001 \); Figure 3.4C), with significant increases in PN burst amplitude (13.94 ± 1.70 vs. 16.54 ± 2.05 µV, \( p<0.001 \)). KCN also increased both the cVN inspiratory (\( \Delta: 14.34 \pm 1.68\% \), \( p<0.001 \)) and post-I activities (\( \Delta: 26.20 \pm 4.19\% \), \( p<0.001 \)) and evoked a significant increase in AbN activity (\( \Delta: 86.31 \pm 11.98\% \), \( p=0.024 \)). The AbN response was initially characterized by the presence of peaks of activity during post-I and late-E phases, which decreased to only late-E bursts as AbN discharge returned to the initial conditions (Figure 3.4A, B).
The inhibition of KF with isoguvacine microinjections notably modified the pattern of chemoreflex respiratory response to KCN (Figure 3.4A, B). The effect of KF inhibition on KCN-induced tachypneic response was variable among experiments. The magnitude of reflex tachypnea remained unchanged in some preparations (N=7 Figure 3.4A) whilst others showed a reduction in respiratory frequency in response to KCN (N=6; Figure 3.4B) compared to the control response. On average, the magnitude of elevation in PN burst frequency in response to peripheral chemoreceptor stimulation was significantly reduced after KF inhibition (Δ frequency: 8.33 ± 0.92 vs. 2.12 ± 2.28 bpm, *p*=0.03, Figure 3.4C). Irrespective of the pattern of change in PN burst frequency, KCN still evoked an increase in PN burst amplitude after KF inhibition (Δ PN: 19.17 ± 4.39 vs. 28.52 ± 4.26 %, *p*=0.114, Figure 3.4D). The increase in cVN inspiratory activity after KCN stimulation was maintained after KF inhibition (Δ cVN I: 14.34 ± 1.68 vs. 17.52 ± 2.35 %, *p*=0.111, Figure 3.4E), but the increase in cVN post-I activity (Δ cVN post-I: 26.20 ± 4.19 vs. 7.65 ± 2.81 %, *p*=0.010, Figures 3.4E) was eliminated. Moreover, the amplitude of AbN activity increased significantly (Δ AbN: 86.31 ± 11.98 vs. 121.54 ± 14.49 %, *p*=0.006, Figure 3.4F) after KF inhibition and was characterized by a tonic burst during the expiratory period (Figure 3.4A, B).

3.3.3 Effect of KF inhibition via isoguvacine on the hypercapnic response

Upon exposure to hypercapnia (N=10), there was a significant increase in the AbN activity during the late-E phase (Figure 3.5A). The emergence of these AbN late-E bursts was accompanied by: i) no significant changes in PN burst frequency (15±2 vs. 13±1 bpm, Figure 3.5B) or amplitude (12.0±1.7 vs. 11.8±1.7 µV); ii) a significant reduction in inspiratory time (1.06 ± 0.08 vs. 0.86 ± 0.08 s; *p*=0.029, Figure 3.5C) and a significant reduction in expiratory
time (4.0±0.3 vs. 3.1±0.2 s, p=0.012); and iii) a significant increase in the cVN post-I peak
(1.27±0.11 vs. 2.39±0.23 µV, p=0.005) and a tendency to increase the cVN post-I duration (1.27
± 0.11 vs. 2.39 ± 0.23 s, p=0.069).

The inhibition of the KF during hypercapnia caused a significant decrease in PN burst
frequency (10.48 ± 0.68 vs. 15.30 ± 1.76 bpm and 13.28 ± 0.80 bpm, p<0.001; Figure 3.5B)
compared to baseline conditions and hypercapnic conditions prior to KF inhibition, due to an
increase in inspiratory time (1.37 ± 0.09 vs 1.06 ± 0.08 sec and 0.86 ± 0.08 sec, p<0.001, Figure
3.5C). Inhibition of the KF had no significant effect on PN amplitude (Figure 3.5D).

Microinjections of isoguvacine in the KF markedly depressed cVN post-I activity (∆ from
baseline: 25.34 ± 5.40 vs. -78.49 ± 6.34 %, p<0.001, Figure 3.5E). Interestingly, although the KF
inhibition did not significantly modify the amplitude of hypercapnia-induced AbN activity (∆
from baseline: 132.49 ± 26.86 vs. 171.05 ± 36.66 %, p=0.239, Figure 3.5F), the duration of the
late-E bursts increased as a result of the earlier onset of late-E bursts relative to PN bursts (1.27 ±
0.36 vs. 2.39 ± 0.74 sec, p<0.001, Figure 3.5G). After the recovery from the inhibition of the KF,
while still being exposed to hypercapnia, the PN, cVN and AbN responses were comparable to
control hypercapnic conditions (Figure 3.5B-F).

3.4 Discussion

Breathing is the product of the communication between multiple specialized neuronal
networks within the brainstem. To date, most research conducted on the involvement of the pons
in generating or modifying the breathing pattern has focused on its role in triggering the
commencement of post-inspiratory (post-I) activity and the inspiratory off-switch (see Mörschel
and Dutschmann, 2009 for review). Here we show that descending inputs from the pons,
specifically the Kölliker-Fuse nucleus (KF), restrain the onset of late-E abdominal nerve activity and thus act as a timekeeper for the active expiratory pattern observed after stimulation of peripheral O2 chemoreceptors and under hypercapnic conditions. This highlights the role of the pons in coordinating active expiration (AE) and outlines the complexity and multi-functionality of the pontine and medullary centers in controlling breathing.

### 3.4.1 The role of the KF in the control of the expiratory phase during eupnea

It has been suggested that the three-phase eupneic breathing pattern relies on an inhibitory ring comprised of the post-inspiratory (post-I) and augmenting-expiratory (aug-E) neurons of the BötC and the inspiratory (I) neurons of the preBötC (Lindsey et al., 1987; Ezure and Manabe, 1988; Smith et al., 2007). Although this inhibitory ring appears to be the kernel of central respiratory pattern and rhythm generation (Molkov et al., 2010), studies indicate that its function depends on synaptic interactions of BötC and preBötC with other respiratory compartments of the brainstem and the pons (Smith et al., 2007; Janczewski et al., 2013).

There is growing evidence to support the role of the pons in generating post-I activity. Dutschmann and Herbet (2006) found that pharmacological inhibition of the KF with isoguvacine eliminated recurrent laryngeal nerve post-I activity and reduced upper airway resistance during expiration. Smith et al., (2007) and Abdala et al., (2009) have also shown that complete transection of the pons, which causes a shift from a three-phase (I, post-I, E) to a two-phase breathing cycle (I, E), eliminated the BötC post-I neuronal activity and the post-I phases of the cVN and AbN. Recently, Bautista and Dutschmann (2014) found that inhibition of the KF resulted in a significant decrease in hypoglossal motor activity during the inspiratory and post-I phases. There is a clear reciprocal inhibition of the post-I neurons with the aug-E neurons.
et al., 2009), and the pons appears to play a role in coordinating the activity of these two neuronal groups. These studies provide overwhelming support of the role of the KF in acting as the inspiratory-off switch, providing tonic drive to excite the post-I neuron activity and thus the termination of I neuron activity, contributing to the functioning of the Bötc/preBötc inhibitory ring.

From our data, inhibition of the KF decreased cVN post-I activity and increased inspiratory motor activity (duration and amplitude), highlighting the importance of the KF in instigating the initiation of the post-I activity and the transition from I to post-I. Bautista and Dutschmann (2016) have shown that KF inhibition does not modify basal AbN activity, suggesting that the KF is not required for the transition from post-I to the second phase of expiration (E2) under eupneic conditions. In this study, there was no difference in baseline AbN activity before or after inhibition of the KF, providing further support to previous observations that the KF is not essential for the transition from post-I to E2 during eupnea – an event that must rely mainly on intrinsic properties of adapting post-I neurons of the Bötc (Paton, 1996; Molkov et al., 2010).

3.4.2 The role of the KF in the control of late-E activity during peripheral and central chemoreflex activation

Under eupneic conditions, tonic abdominal expiratory activity is low and expiratory flow is generated passively by recoil forces of the lungs and the chest (Jenkin and Milson, 2014). Of the studies that have investigated active expiration (AE) with increasing respiratory drive, AE typically appears at the end of the expiratory (late-E) pause (Sherrey et al., 1988; Taccola et al., 2007; Abdala et al., 2009; Pagliardini et al., 2011). With greater respiratory drive, AE begins
earlier, occupying more of the expiratory phase (Gautier et al., 1973; Sherrey et al., 1988; De Troyer et al., 1989). The generation of late-E abdominal activity depends on the activation of the RTN/pFRG (Janczewski and Feldman, 2006), which contains expiratory neurons that are silenced under resting conditions due to tonic inhibitory drive (Pagliardini et al., 2011). Under hypoxic or hypercapnic conditions, it has been suggested that the RTN/pFRG late-E activity emerges due to increased excitatory and reduced inhibitory drives to the RTN/pFRG (Abdala et al., 2009; Marina et al., 2010; Moraes et al., 2012) and provides excitatory inputs to the ventral respiratory column (Molkov et al., 2010).

We observed that peripheral chemoreceptor stimulation by KCN evoked an increase in respiratory frequency, vagal post-I activity, and post-I abdominal and late-E abdominal motor activity, consistent with a number of previous studies. When the KF was inhibited, the increase in vagal post-I activity was largely depressed and the abdominal response was potentiated and occupied the entire expiratory phase. The tachypneic response was also greatly attenuated. Given that the post-I neurons of the BötC are considered a source of inhibition of the RTN/pFRG (Abdala et al., 2009), we conclude that O₂ chemoreflex-induced post-I activity restrains the emergence of late-E activity. With the inhibition of the KF, late-E activity was released from inhibition (consequent to post-I depression) and the excitatory inputs, potentially from the NTS (Takakura et al., 2006; Moraes et al., 2012), generated an amplified abdominal response. Furthermore, considering that the recruitment of RTN/pFRG late-E neurons is associated with enhanced BötC aug-E neuron activity (Abdala et al., 2009), the augmented late-E neuron activation after KF inhibition may also contribute to the observed expiratory lengthening during peripheral chemoreceptor stimulation, preventing the tachypneic reflex response. Our data provide new evidence that the KF critically regulates the post-I activity during peripheral
chemoreflex activation, which controls the duration of abdominal late-E activity and the magnitude of the increase in respiratory frequency.

Under hypercapnic conditions, abdominal activity appears to emerge as a result of RTN/pFRG late-E neuronal activation (Janczewski and Feldman, 2006; Abdala et al., 2009; Molkov et al., 2010) due to the stimulation of CO₂-chemosensitive neurons (Marina et al., 2010). Interestingly, post-I expiratory breaking activity is always conserved in the breathing cycle, and thus late-E activity is always preceded by post-I activity (Sherrey et al., 1988; Mörschel and Dutschmann, 2009). This suggests that the transition from passive to active expiration is likely controlled by the termination of post-I neuron activity. Abdala et al., (2009) demonstrated that the abdominal late-E bursts during hypercapnia (10% CO₂) occur in parallel with reductions in vagal post-I activity. Our results show that vagal post-I duration decreases, albeit not significantly, with the presence of late-E AE. The lack of significance is probably due to the use of lower CO₂ levels (8% CO₂). When the KF was inhibited, post-I activity was further depressed and AbN late-E activity was amplified. Consequently, expiratory time was prolonged, potentially by increased activity of BötC aug-E neurons (see Abdala et al., 2009) and respiratory frequency was decreased. Consistent with this, despite the inhibition of inspiration and production of post-I activity in the cVN, stimulation of the KF with glutamate did not produce AE (Figure 3.1). In conjunction, our data support that the KF-dependent post-I neuronal activity inhibits the mechanisms generating AE in hypercapnia. Additional studies are still required to identify how KF neurons interact with the neural network involved with the generation of active expiration. However, two new hypotheses are raised: 1) the KF may be inhibited during hypercapnia, causing depression of tonic drive to BötC post-I neurons; or 2) the BötC may be inhibited during hypercapnia, and KF tonic drive may maintain post-I activity during this period of inhibition.
3.4.3 Conclusion

The pons, specifically the KF, is considered to be the inspiratory off-switch, triggering the start of post-I neuron activity and consequent inhibition of inspiratory neuronal activity. Our study concludes that the KF also plays a role in inhibiting the emergence of the late-E abdominal activity under conditions of metabolic challenge, acting as a timekeeper for active expiration. While the mechanisms that allow the pons to control the duration of late-E AbN activity remain unknown, it is clear that the KF is critical in determining the duration of late-E activity observed during hypercapnia and hypoxia. Our data extend the current knowledge about the respiratory central pattern generator and open new possibilities to understand the development of cardiorespiratory changes associated with pathological conditions of central and peripheral chemoreceptor hyperactivity, such as sleep breathing disorders, neurogenic hypertension and heart failure.
Figure 3.1 Raw and integrated (∫) recordings of the abdominal (AbN), cervical vagus (cVN), and phrenic nerve (PN) activities of an in situ preparation, representative from the group, illustrating the pattern of respiratory changes elicited by unilateral microinjection of glutamate (10mM) into the KF (arrow).
Figure 3.2 Panel A: Bright-field (top) and fluorescent (bottom) microscope images of brainstem coronal sections of a representative *in situ* preparation, showing the injection site of isoguvacine into the KF. Panel B: schematic representation of all injection (N=13) sites into the KF.

Abbreviations: DLL – dorsal nucleus of the lateral lemniscus; scp – superior cerebellar peduncle; s5 – sensory root of trigeminal nerve.
Figure 3.3 Raw and integrated (\(\int\)) recordings of the abdominal (AbN), cervical vagus (cVN), and phrenic nerve (PN) activities of an *in situ* preparation, representative from the group, showing the respiratory responses to bilateral injections of the isoguvacine (arrows) into the KF. The two injections were completed within two minutes, and after the second injection there was a reduction the cVN post-inspiratory activity. These effects lasted between 1 and 5 minutes, after which there was a complete recovery of the cVN post-inspiratory activity. Average values are presented for PN burst frequency (B) and amplitude (C), time of inspiration (D) and expiration (E), cVN inspiratory (F) and post-inspiratory (G) activity and AbN activity during E1 (H) and E2 (I). Bars represent means \(\pm\) S.E.M. N=13, * represents \(p<0.05\).
Figure 3.4 Raw and integrated (∫) recordings of cervical vagus (cVN), abdominal (AbN) and phrenic nerve (PN) activities of an *in situ* preparation, representative from the group, showing the increase in breathing frequency upon peripheral chemoreflex activation (with KCN, arrows) before and after the inhibition of KF with bilateral microinjections of isoguvacine (10 mM, ISO arrow). Representative traces of the tachypnea effect observed by 7 rats upon KCN stimulation after KF inhibition (A) and the bradypnea effect observed by 6 rats upon KCN stimulation after KF inhibition (B) are both shown. Average values are presented for PN burst frequency under basal and after stimulation with KCN observed before KF inhibition and after KF inhibition (B), the percent change in PN amplitude after KCN before and after KF inhibition (C), the percent change in both inspiratory and post-I cVN activity after KCN before and after KF inhibition (D), and the percent change in AbN amplitude after KCN before and after KF inhibition (E). Bars represent means ± S.E.M. N=13, * represents *p*<0.05.
Figure 3.5 The raw and integrated (∫) trace of abdominal (AbN), cervical vagus (cVN), and phrenic nerve (PN) activities from an in situ preparation, representative of the group, showing the pattern of respiratory motor activities under baseline conditions (5% CO₂, 95% O₂) and under hypercapnic conditions (8% CO₂, 92% CO₂) before, after KF inhibition with isoguvacine (10 mM) and after recovery from the KF inhibition. Note that after KF inhibition, cVN post-I activity decreased and the onset of the AbN late-E burst increased. Average values are presented for PN burst frequency (B) and time of inspiration (C) under basal conditions and after stimulation with hypercapnia observed before KF inhibition and after KF inhibition (ISO) and after recovery from KF inhibition. Additionally, the percent change in PN amplitude (D), cVN amplitude (E), Abdominal amplitude (F) and ABN onset (G) between baseline conditions and during hypercapnic conditions before KF inhibition, during KF inhibition (ISO) and after recovery from KF inhibition are presented. Bars represent means ± S.E.M. N=10, * represents $p<0.05$. 
Chapter 4: The hypercapnic ventilatory response in neonatal rats: the balance between factors intrinsic and extrinsic to the medulla

4.1 Introduction

Total ventilation ($\dot{V}_E$) is the result of a balance between tidal volume ($V_T$) and breathing frequency ($f_R$). The contribution of each of $V_T$ and $f_R$ to $\dot{V}_E$ reflects the balance of multiple inputs acting on the central rhythm generators that set the frequency of the inspiratory and expiratory motor output (Wyman, 1977). These include inputs from peripheral and central chemosensors as well as from pulmonary stretch receptors in the lungs and descending input from the pons (Stella, 1983; Heck and Levitzky, 2008; Mörschel and Dutschmann, 2009; Dutschmann and Dick, 2012). Hypercapnia (high levels of inspired CO$_2$) is a respiratory stimulant that increases respiratory drive by activating peripheral and central chemoreceptors to increase $\dot{V}_E$ via increases in $V_T$ and $f_R$. The corresponding changes of each of these variables differ between species as well as within species as a function of the level of hypercapnia, the time course of the exposure and the developmental age of the individual (Putnam et al., 2005). In adult rats, the increase in $\dot{V}_E$ with increasing levels of CO$_2$ (the hypercapnic ventilatory response (HCVR)) is often due to an immediate increase in $f_R$ with a more slowly developing increase in $V_T$ (Lai et al., 1978). The proportionate increase in $V_T$ predominates and appears to be greater the larger the increase in CO$_2$ (Cragg and Drysdale, 1983; Mouradian et al., 2012). The situation in neonatal rats is more complex. During the first day of life, rat pups exhibit a relatively large increase in $\dot{V}_E$ in response to increases in inspired CO$_2$ (Stunden et al., 2001; Wickström et al., 2002). In most studies, over the next few days CO$_2$ sensitivity declines to a minimum at postnatal day 8 (P8) (Stunden et al., 2001; Serra et al., 2001; Wickstrom et al., 2002) and then rises.
again, stabilizing at adult levels by P21. Most investigators report that the increase in $\dot{V}_E$ seen at birth is due either largely or entirely to increases in $V_T$. Increases in $f_R$, if present, disappear in the first few days of life and then return slowly giving rise to the adult pattern of responsiveness (Bamford et al., 1996; Saiki and Mortola, 1996; Abu-Shawesh et al., 1999; Studden et al., 2001; Serra et al., 2001; Wickström et al., 2002; Davis et al., 2006).

The developmental changes seen in rat pups in their ventilatory sensitivity to CO$_2$ may arise from developmental changes at multiple sites ranging from changes in chemoreceptor input, central integration of that input or the production of motor output. This makes it difficult to identify the mechanisms that contribute to any changes in $V_T$ or $f_R$ definitively using in vivo studies. In this study, we characterized developmental changes in the HCVR restricted to the pons and medulla of neonatal rats during the first few days of life using the en bloc brainstem-spinal cord preparation as first described by Suzue (1984). This preparation contains the functional respiratory rhythm generating sites, the functional circuitry between the pons and medulla, and the pre-motor and motor outputs to the spinal nerves that innervate the respiratory muscles. The preparation is superfused with oxygenated artificial cerebrospinal fluid (aCSF) and diffusion of oxygen from the circulating aCSF to the respiratory centres is adequate to maintain respiratory activity (Fong et al., 2008). The preparation is responsive to increased CO$_2$ (Kawai et al., 1996), and permits investigation of central elements of the HCVR in the absence of peripheral inputs and descending influence from higher brain centres. We hypothesized that if developmental changes in central synaptic mechanisms underlie the reduction in the HCVR (i.e. the decrease in the $V_T$ response) seen in rat pups during the first 8 days of life, then we would see a reduction in the total burst activity in respiratory motor output associated with each fictive breath from the in vitro preparation.
4.2 Methods

All experiments were performed with approval from the University of British Columbia Animal Care Committee (A13-0025), under the guidelines of the Canadian Council for Animal Care (CCAC).

4.2.1 Experimental animals

All experimental trials were performed in vitro on en bloc brainstem-spinal cord preparations from newborn Sprague-Dawley rats (0-4 days postnatal (P0-P4)). Preparations were either ponto-medullary (medulla and pons) (n=7) or medullary (medulla alone) (n=7) for each age group (P0, day of birth; P2, 1-2 days postnatal; P4, 3-4 days postnatal).

4.2.2 The in vitro brainstem spinal cord preparation

Rat pups were anesthetized with ~2% isoflurane until the toe pinch reflex was abolished. The brainstem and spinal cord were isolated en bloc (as described by Suzue, 1984) at room temperature while submerged in artificial cerebral spinal fluid (aCSF) (1.5 mM CaCl₂, 113.0 mM NaCl, 9.0 mM KCl, 1.2 mM NaH₂PO₄, 30.0 mM NaHCO₃, 30.0 mM D-glucose). The brainstem was transected at one of two locations: (1) just rostral of the pons for a ponto-medullary-spinal cord preparation, or (2) just caudal to the anterior inferior cerebellar artery for a medullary-spinal cord preparation (as described by Fong et al., 2008). The full spinal cord was isolated in each preparation. The entire en bloc preparation was placed ventral side up in a recording dish. The preparation was maintained at 27°C by a Lauda water bath (Model RC6) and superfused with 95% O₂ and 5% CO₂ aCSF. Whole nerve activity was simultaneously recorded from the 4th
cervical (C4) rootlet and the 11th thoracic (T11) rootlet with glass suction electrodes. The activity was amplified, filtered (0.10Hz-3kHz) and recorded at 2kHz using a Windaq data acquisition system (D1200; Data Instruments, USA; as described by Fong et al., 2008).

After a 20-minute exposure to the 95% O₂ and 5% CO₂ aCSF, the preparations from the three different age groups were exposed to three levels of O₂/CO₂: 100/0%, 95/5%, and 90/10% in random order for 20 minutes each.

4.2.3 Data and statistical analysis

All data were recorded using the Windaq acquisition hardware and software (D1200; Data Instruments, USA) and then analyzed using Spike2 (5.08; Cambridge Electronics Design, UK). Raw nerve recordings were integrated for measurements of burst area (volts·sec) and fictive breathing frequency for both C4 and T11 motor nerve output (as described in Fong et al., 2008). Due to the variability in burst frequency and burst amplitude between preparations, percent changes in burst area and fictive breathing frequency were calculated from the absolute changes in burst area and fictive breathing frequency. The inspiratory interval (T₁), expiratory interval (Tₑ) were measured from the C4 motor nerve output and the difference in onset and offset time of the T11 motor nerve output relative to the C4 motor nerve output was also determined from five-minute sections at the end of each CO₂ exposure.

All data were analyzed for significant differences with SigmaStat 11 (Dundas Software LTD, Germany). Statistical significance for the effect of increased CO₂ and age on the percent change in burst area and fictive breathing frequency was determined using a repeated measures ANOVA. Respiratory durations of the motor output (T₁, Tₑ, and differences in burst onset and burst offset) at different ages and different CO₂ levels within each preparation type (ponto-
medullary and medullary) were also compared with a one-way ANOVA. Values for these variables between ponto-medullary and medullary preparation were compared with t-tests. If normality or equal variance was not passed the ANOVA was calculated on ranks. Holm-Sidak or Tukey Tests were used as appropriate, $p<0.05$ was considered significant in all cases.

4.3 Results

4.3.1 Effect of CO$_2$ on burst area (indicative of tidal volume)

As CO$_2$ levels increased from 0% to 10%, C4 burst area decreased by 30.4±16.5% for P0, by 33.7±9.2% for P2, and by 38.7±12.8% for P4 ponto-medullary preparations (Figure 4.1A). This decrease was significant for older preparations, with C4 motor output burst area decreasing in P4 preparations between 0% CO$_2$ and 5% CO$_2$ ($p=0.015$) and between 0% CO$_2$ and 10% CO$_2$ ($p=0.008$) (Figure 4.1A). Similarly, as CO$_2$ levels increased from 0% to 10%, T11 burst area decreased by 25.2±23.6% for P0, by 33.4±14.2% for P2, and by 53.4±10.4% for P4 preparations. This decrease was also significant for older preparations, with T11 motor output burst area decreasing in P4 preparations between 0% CO$_2$ and 5% CO$_2$ ($p=0.009$), between 0% CO$_2$ and 10% CO$_2$ ($p<0.001$) and between 5% CO$_2$ and 10% CO$_2$ ($p=0.046$) (Figure 4.1B).

A similar effect was observed for the medullary preparations (Figure 4.1C, D). As CO$_2$ levels increased from 0% to 10%, burst area decreased by 32.2±17.0 % for P0, 34.6±5.5% for P2, and by 19.2±8.0% for P4 preparations. This decrease was significant for older preparations, with C4 motor output burst area decreasing in P2 preparations between 0% CO$_2$ and 10% CO$_2$ ($p=0.005$) and in P4 preparations between 5% CO$_2$ and 10% CO$_2$ ($p=0.003$) (Figure 4.1C). As CO$_2$ levels increased from 0% to 10% CO$_2$, T11 burst area decreased by 42.1±15.6% for P0, by 30.2±14.6% for P2 and by 53.1± 4.1% for P4 medullary preparations. This decrease was
significant for P0 preparation between 0% CO\textsubscript{2} and 10% CO\textsubscript{2} ($p=0.017$), and for P4 preparations between 0% CO\textsubscript{2} and 5% CO\textsubscript{2} ($p=0.005$), between 0% CO\textsubscript{2} and 10% CO\textsubscript{2} ($p<0.001$) and between 5% CO\textsubscript{2} and 10% CO\textsubscript{2} ($p<0.001$) (Figure 4.1B) (Figure 4.1D).

### 4.3.2 Effect of CO\textsubscript{2} on fictive breathing frequency

As CO\textsubscript{2} increased from 0% to 10%, C4 burst frequency increased by $100.4\pm34.7\%$ for P0, by $109.8\pm137.2\%$ for P2, and by $45.8\pm30.3\%$ for P4 ponto-medullary preparations (Figure 4.2A). This increase was significant for P0 preparations, with fictive breathing frequency increasing between 0% CO\textsubscript{2} and 10% CO\textsubscript{2} ($p=0.036$) and for P4 preparations between 0% CO\textsubscript{2} and 10% CO\textsubscript{2} ($p=0.028$; Figure 4.2A). Similarly, T11 burst frequency increased by $105.1\pm36.9\%$ for P0, by $65.8\pm19.2\%$ for P2, and by $39.3\pm31.24\%$ for P4 ponto-medullary preparations. This increase was significant for P0 preparations, with fictive breathing frequency increasing between 0% CO\textsubscript{2} and 10% CO\textsubscript{2} ($p=0.010$, Figure 4.2B).

A similar trend was observed for the medullary preparations (Figure 4.2C, D). As CO\textsubscript{2} increased from 0% to 10%, C4 burst frequency increased by $125.4\pm36.2\%$ for P0, $130.1\pm97.8\%$ for P2, and by $82.2\pm21.6\%$ for P4 medullary preparations. This increase was significant for P0 preparations between 0% CO\textsubscript{2} and 10% CO\textsubscript{2} ($p=0.007$), and for P4 preparations between 0% CO\textsubscript{2} and 10% CO\textsubscript{2} ($p<0.001$) and between 5% CO\textsubscript{2} and 10% CO\textsubscript{2} ($p=0.004$, Figure 4.3C). Similarly, as CO\textsubscript{2} increased, T11 burst frequency increased by $172.8\pm55.8\%$ for P0, by $121.4\pm98.2\%$ for P2 and by $58.2\pm17.1\%$ for P4 medullary preparations. This increase was significant in P4 preparations between 0% CO\textsubscript{2} and 10% CO\textsubscript{2} ($p=0.002$) and between 5% CO\textsubscript{2} and 10% CO\textsubscript{2} ($p=0.004$, Figure 4.2D).
4.3.3 Effect of CO$_2$ on inspiratory and expiratory time

As CO$_2$ increased, there was a tendency for $T_I$ to decrease for both C4 and T11 motor nerve output in ponto-medullary preparations. This decrease was significant for the C4 motor output of P4 preparations between 0% CO$_2$ (1.03±0.17 sec) and 5% CO$_2$ (0.74±0.11, $p=0.014$) and between 0% CO$_2$ (1.03±0.17 sec) and 10% CO$_2$ (0.63±0.13, $p=0.002$) and for the T11 motor output of P4 preparations between 0% CO$_2$ (0.89±0.13 sec) and 10% CO$_2$ (0.48±0.09 sec; $p=0.003$, Table 4.1A). This was also true for medullary preparations, where the decrease was significant in P4 preparations for C4 motor output between 0% CO$_2$ (0.99±0.15 sec) and 10% CO$_2$ (0.73±0.06 sec, $p=0.017$) and between 5% CO$_2$ (0.97±0.08 sec) and 10% CO$_2$ (0.73±0.06 sec, $p=0.024$) and for T11 motor output between 0% (0.96±0.14 sec) and 10% (0.59±0.14 sec, $p=0.003$, Table 4.1B).

As CO$_2$ increased, there was also a tendency for $T_E$ to decrease for both C4 and T11 motor output for ponto-medullary preparations. However, these decreases only became significant in P4 preparations. $T_E$ of C4 motor output in P4 preparations decreased as CO$_2$ increased from 0% (19.69±5.73 sec) to 10% (7.36±1.87 sec, $p=0.001$, Table 4.1A). There was also a significant decrease in $T_E$ for T11 motor output in P4 preparations as CO$_2$ increased from 0% (20.04±6.25 sec) to 10% CO$_2$ (7.05±1.57 sec, $p=0.001$, Table 4.1A). In medullary preparations, CO$_2$ levels appeared to have even less of an effect on $T_E$. While the trend was the same as it was for the ponto-medullary preparations, there was only a significant change in $T_E$ for T11 motor output in P4 preparations as CO$_2$ increased from 5% (14.55±2.71) to 10% (8.22±1.36, $p=0.016$, Table 4.1B).
4.3.4 The effect of age and the pons on respiratory – related motor output

There was a significant effect of age on $T_I$ and $T_E$ at higher levels of CO$_2$ exposures. At 5% CO$_2$, there was a significant decrease in C4 $T_I$ between P0 (1.35±0.143) and P4 (0.74±0.11, $p=0.009$). Similarly, at 5% CO$_2$, there was a significant decrease in T11 $T_I$ between P0 (1.89±0.40) and P4 (0.66±0.078, $p=0.003$). With medullary preparations, the effect across age groups was observed at higher levels of CO$_2$, with C4 $T_I$ decreasing at 10% between P0 (1.23±0.11) and P4 (0.73±0.06; $p=0.002$) and for T11 $T_I$ at 10% between P0 (1.05±0.10) and P4 (0.59±0.14; $p=0.014$). There was little effect of age on $T_E$, except for T11 $T_E$ between P2 (13.09±5.32) and P4 (8.22±1.36) at 10% CO$_2$ ($p=0.027$) in medullary preparations.

4.3.5 The effect of CO$_2$, age and the pons on C4-T11 burst coordination

Typically, bursts of activity in T11 began shortly after the C4 bursts and this delay increased with CO$_2$ level and with age. It was only significant, however, in medullary P4 preparations between 0% CO$_2$ (0.02±0.05 sec) and 10% CO$_2$ (0.18±0.03 sec, $p=0.002$, Figure 4.3B). Termination of T11 burst also tended to occur before the end of the C4 burst, but this difference was not significant between age groups or CO$_2$ levels with either ponto-medullary or medullary preparations (Figure 4.3A, B).

4.4 Discussion

The in vivo HCVR observed in neonatal rats changes greatly over development. P0 rat pups exhibit a large increase in $V_E$ due consistently to a large increase in $V_T$, with little or no change in $f_R$ (Putnam et al., 2005). During the first week of life (P1-P8), the HCVR decreases due to a substantial decrease in the $V_T$ response (Cragg and Drysdale, 1983; Saetta and Mortola,
but see Huang et al., 2010). Increases in breathing frequency ($f_R$), if present, disappear in the first few days of life and then return slowly giving rise to the adult pattern of responsiveness (Bamford et al., 1996; Saiki and Mortola, 1996; Abu-Shawesh et al., 1999; Stunden et al., 2001; Serra et al., 2001; Wickström et al., 2002; Davis et al., 2006). We used the en bloc brainstem-spinal cord preparation to determine the extent to which the changes in the in vivo HCVR exhibited by neonatal rats over the first four days of life are due to changes intrinsic to the pons and medulla. We found that increasing the levels of CO$_2$ in the artificial cerebrospinal fluid (aCSF) surrounding the pons and medulla produced a decrease in the fictive $V_T$ (as represented by C4 and T11 burst area) that became progressively greater between P0 and P4. It also led to an increase in fictive $f_R$. These results indicate that while developmental changes are occurring in the pons and medulla that affect the HCVR, the maturational changes in the HCVR seen in vivo are also shaped by both excitatory and inhibitory factors extrinsic to the pons and medulla.

### 4.4.1 Effect of CO$_2$ on burst area (fictive $V_T$)

Increasing levels of CO$_2$ in the aCSF almost never stimulated an increase in fictive $V_T$ in vitro even at P0 in the present study. 5% CO$_2$ produced a non-significant increase in the burst area of T11 discharge in P0 and P2 ponto-medullary preparations, and in the burst area of C4 discharge in P0 and P4 medullary preparations. Thus, the large increase in $V_T$ reported at P0/P1 in vivo must come from somewhere else, as only a progressive reduction in fictive $V_T$ is observed in vitro. Thus, while the progressive decrease in fictive $V_T$ seen here may account for (or, at least, contribute to) the reduction in the $V_T$ response seen in vivo (Stunden et al., 2001), excitation from some other source is required to produce the initial increase in $V_T$ that this acts
on. A similar (albeit insignificant) trend to the initial increase and subsequent decrease in the $V_T$ response seen in vivo over the first few days of life is also seen in decerebrate (brainstem transected at the pre-collicular level), vagotomized rat pups (Zhou et al., 1996) suggesting that the absence of the initial increase in $V_T$ is not due to the absence of the cerebrum or vagal input. A primary difference between the decerebrate, vagotomized preparation and the in vitro preparation used here is the absence of peripheral chemoreceptor feedback from the carotid bodies suggesting that this may be the source of the input leading to the increase in $V_T$. There were no significant differences between the results obtained from the ponto-medullary and medulla only preparations in the present study, suggesting that the reduction in the $V_T$ response arises from changes occurring within the medulla itself. That it lies outside the rhythm generating networks is suggested by the observation that elevating CO$_2$ levels of the aCSF in the in vitro transverse brain slice, an even further reduced preparation, results in an increase in fictive $f_R$, with no change in burst amplitude of respiratory-related motor output in the hypoglossal nerve of P1-P4 rat pups (Peever et al., 2001).

It is unlikely that the decrease in $V_T$ seen with the in vitro brainstem-spinal cord preparation was due to a decrease of the viability of the preparation. While we did observe a decrease in burst area with increasing levels of CO$_2$, the different levels of CO$_2$ were randomized for each preparation. We also observed a concurrent increase in burst frequency.

### 4.4.2 Effect of CO$_2$ on fictive breathing frequency

As noted above, the in vivo HCVR observed in neonatal rats there is little or no change in $f_R$ associated with the HCVR in neonatal rats during the first week of life (Putnam et al., 2005). Indeed, in some studies it has been shown that both unrestrained Sprague-Dawley rat pups, as
well as ventilated, decerebrate, vagotomized, paralyzed rat pups, decrease breathing frequency significantly at P5 but increase it significantly at P16 and beyond (Abu-Shaweesh et al., 1999). In the current study, elevating CO$_2$ levels of the aCSF of in vitro brainstem-spinal cord preparations resulted in an increase in fictive $f_R$ (recorded from two inspiratory nerves, the C4 and the T11) in P0-P4 rat pups. With the in vitro transverse brain slice from P1-P4 rat pups, elevating CO$_2$ levels of the aCSF also resulted in an increase in the fictive $f_R$, (recorded from the hypoglossal nerve; Peever et al., 2001). These observations suggest both that CO$_2$ acts directly on elements present in the transverse slice to excite $f_R$ and that this excitation is over-ridden by inhibition from some other (more peripheral) source to produce the decrease in $f_R$ seen in vitro.

4.4.3 The effect of CO$_2$, age and the pons on C4-T11 burst coordination

The observed change in $f_R$ associated with the HCVR in adult rats in vivo is often due to both a decrease in T$_I$ and T$_E$, however, the decrease in T$_E$ is often more pronounced (Cragg and Drysdale, 1983). This was also true for the increases in $f_R$ seen with our in vitro data. As T$_E$ made up such a large proportion of total breath time (T$_{TOT}$), decreases in T$_E$ contributed more substantially to the overall increase in breathing frequency (Table 4.1).

The two nerves recorded from in the present study were the 4$^{th}$ cervical (C4) and the 11$^{th}$ thoracic (T11) spinal motor nerves. The C4 branches to become the phrenic nerve, which innervates the diaphragm (Peever and Duffin, 2001) and the T11 nerve branches to innervate the intercostal muscles and the abdominal muscles (Giraudin et al., 2008). As the C4 and T11 bursts were always synchronized in our study, they are both considered inspiratory motor nerves, the latter likely innervating external intercostal muscles. There was a slight delay in T11 burst onset compared to the C4 burst onset, consistent with the rostrocaudal gradient in burst onset observed
using multiple simultaneous thoracic motorneuron recordings (Giraudin et al., 2008). The delay between the onset of the T11 burst compared to its C4 burst was exaggerated in our medullary preparations compared to the ponto-medullary preparations, suggesting that descending pontine control plays a role in coordinating the onset of these motorneuron bursts. The delay was also longer in older preparations; the T11 burst offset was significantly longer in P4 than P0 preparations (Figure 4.3B). Interestingly, not only did the T11 motor neuron burst start later, there was a trend for the T11 burst duration to shorten. This would suggest that the T11 intercostal motor output was of greater importance in powering breathing in younger animals, and at lower levels of respiratory drive.

4.4.4 Conclusion

Exposure to hypercapnia in P0 to P4 rat pups in vitro produced a decrease in tidal volume (represented by C4 and T11 motor neuron burst area) and an increase in fictive breathing frequency. These data suggest that the HCVR observed in vivo consists of an initial increase in $V_T$, possibly due to peripheral chemoreceptor stimulation, that is progressively inhibited by changes occurring within the medulla that lie outside the rhythm generating networks. It also consists of an excitation of $f_R$ arising from elements present in, or in the vicinity of the rhythm generating circuits and inhibition arising from more peripheral inputs that result in little or no change in $f_R$. Also, we show that descending pontine control plays a role in the coordination of inspiratory spinal motor nerve output, especially in older preparations. This highlights the importance of the intercostal muscles early in development and in the role of the pons in coordinating the respiratory muscles.
Figure 4.1 Effect of age and CO$_2$ levels on the fictive tidal volume of C4 and T11 motor nerve output from ponto-medullary and medullary preparations. Percent change in burst area (representing tidal volume) of (A) C4 motor nerve activity (blue bars) and (B) T11 motor nerve activity (purple) for P0, P2 and P4 age groups exposed to 0%, 5% and 10% CO$_2$ for ponto-medullary preparations. Percent change in burst area for (C) C4 motor nerve activity (blue bars) and (D) T11 motor nerve activity (purple) for P0, P2 and P4 age groups exposed to 0%, 5% and 10% CO$_2$ for medullary preparations. Significant differences between CO$_2$ levels in each age group represented by a *, $p<0.05$. 


Figure 4.2 Effect of age and CO₂ levels on the fictive breathing frequency of C4 and T11 motor nerve output from ponto-medullary and medullary preparations. Percent change in fictive breathing frequency (representing tidal volume) of (A) C4 motor nerve activity (blue bars) and (B) T11 motor nerve activity (purple) for P0, P2 and P4 age groups exposed to 0%, 5% and 10% CO₂ for ponto-medullary preparations. Percent change in fictive breathing frequency for (C) C4 motor nerve activity (blue bars) and (D) T11 motor nerve activity (purple) for P0, P2 and P4 age groups exposed to 0%, 5% and 10% CO₂ for medullary preparations. Significant differences between CO₂ levels in each age group represented by a *, p<0.05.
Figure 4.3 Effect of CO$_2$ and age on the onset and duration of the C4 and T11 motor nerve activity. The difference in onset of the T11 motor output (purple bars) compared to its synchronized C4 motor output (blue bars) for P0, P2 and P4. A) ponto-medullary preparations and B) medullary preparations exposed to 0%, 5% and 10% CO$_2$. Significant differences between CO$_2$ levels at each age group represented by a *, p<0.05.
Table 4.1 The inspiratory (T_I) and expiratory (T_E) for both the C4 motor nerve and T11 motor nerve bursts. Values provided for different age groups (P0, P2, and P4) at different levels of CO₂ (0%, 5%, and 10%) for both ponto-medullary and medullary preparations. Significant differences between CO₂ levels at each age group represented by a *, significant differences between age groups at each CO₂ level are represented by a #, p<0.05.
### Ponto-Medullary

<table>
<thead>
<tr>
<th></th>
<th>P0</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T₁ (sec)</td>
<td>T₂ (sec)</td>
<td>T₁ (sec)</td>
<td>T₂ (sec)</td>
<td>T₁ (sec)</td>
<td>T₂ (sec)</td>
<td>T₁ (sec)</td>
<td>T₂ (sec)</td>
<td>T₁ (sec)</td>
<td>T₂ (sec)</td>
<td>T₁ (sec)</td>
</tr>
<tr>
<td>0%</td>
<td>C4</td>
<td>T₁1</td>
<td>C4</td>
<td>T₁1</td>
<td>C4</td>
<td>T₁1</td>
<td>C4</td>
<td>T₁1</td>
<td>C4</td>
<td>T₁1</td>
<td>C4</td>
</tr>
<tr>
<td></td>
<td>1.36±0.20</td>
<td>1.19±0.20</td>
<td>38.88±10.11</td>
<td>40.17±9.63</td>
<td>1.26±0.075</td>
<td>0.95±0.23</td>
<td>40.79±21.68</td>
<td>28.8±11.63</td>
<td>1.03±0.17</td>
<td>0.89±0.13*</td>
<td>19.69±5.73*</td>
</tr>
<tr>
<td>5%</td>
<td>1.35±0.14*</td>
<td>1.89±0.40</td>
<td>42.78±19.84</td>
<td>43.84±19.80</td>
<td>1.24±0.18</td>
<td>1.23±0.20</td>
<td>32.52±11.48</td>
<td>35.89±3.67</td>
<td>0.74±0.11*</td>
<td>0.66±0.078*</td>
<td>10.89±1.463</td>
</tr>
<tr>
<td>10%</td>
<td>1.24±0.14</td>
<td>1.47±0.34</td>
<td>25.92±9.84</td>
<td>23.54±9.30</td>
<td>1.035±0.077</td>
<td>0.83±0.084</td>
<td>12.79±2.09</td>
<td>13.30±2.01</td>
<td>0.63±0.13</td>
<td>0.48±0.09*</td>
<td>7.36±1.87*</td>
</tr>
</tbody>
</table>

### Medullary

<table>
<thead>
<tr>
<th></th>
<th>P0</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T₁ (sec)</td>
<td>T₂ (sec)</td>
<td>T₁ (sec)</td>
<td>T₂ (sec)</td>
<td>T₁ (sec)</td>
<td>T₂ (sec)</td>
<td>T₁ (sec)</td>
<td>T₂ (sec)</td>
<td>T₁ (sec)</td>
<td>T₂ (sec)</td>
<td>T₁ (sec)</td>
<td>T₂ (sec)</td>
</tr>
<tr>
<td>0%</td>
<td>C4</td>
<td>T₁1</td>
<td>C4</td>
<td>T₁1</td>
<td>C4</td>
<td>T₁1</td>
<td>C4</td>
<td>T₁1</td>
<td>C4</td>
<td>T₁1</td>
<td>C4</td>
<td>T₁1</td>
</tr>
<tr>
<td></td>
<td>1.37±0.39</td>
<td>1.00±0.24</td>
<td>27.51±9.68</td>
<td>28.51±7.48</td>
<td>0.92±0.057</td>
<td>0.78±0.09</td>
<td>14.12±2.40</td>
<td>14.78±2.23</td>
<td>0.99±0.15</td>
<td>0.96±0.14*</td>
<td>15.19±3.89</td>
<td>12.34±1.10</td>
</tr>
<tr>
<td>5%</td>
<td>1.33±0.17</td>
<td>1.15±0.21</td>
<td>22.72±7.75</td>
<td>18.47±7.86</td>
<td>0.92±0.056</td>
<td>0.83±0.11</td>
<td>12.57±2.56</td>
<td>12.67±2.25</td>
<td>0.97±0.08</td>
<td>0.73±0.13</td>
<td>17.04±4.88</td>
<td>14.55±2.71</td>
</tr>
<tr>
<td>10%</td>
<td>1.23±0.11*</td>
<td>1.05±0.10</td>
<td>16.37±5.08</td>
<td>15.67±4.88</td>
<td>0.83±0.12</td>
<td>0.65±0.11</td>
<td>13.45±5.40</td>
<td>13.09±5.32*</td>
<td>0.73±0.06*</td>
<td>0.59±0.14*</td>
<td>8.00±1.28</td>
<td>8.22±1.36*</td>
</tr>
</tbody>
</table>
Chapter 5: Respiratory muscle recruitment under hypercapnic conditions: a comparative analysis in rodents

5.1 Introduction

Under eupneic conditions, the mammalian breathing cycle is typically composed of three phases: active inspiration (I), the first expiratory phase (E1), characterized by a prolonged contraction of the inspiratory muscles that brake the rate at which air is exhaled from the lungs, and a second expiratory phase (E2) during which there is only passive expiration and, depending on the rate of breathing, a pause with no muscle contraction and no airflow (Richter, 1996; Feldman and McCrimmon, 2003). Normally under resting conditions, inspiration is active, and expiration is passive. However, when respiratory drive is elevated there is often recruitment of the abdominal muscles to force air out of the lungs, generating an active expiration (AE) (De Troyer et al., 1989; Feldman et al., 2013).

There are some mammals, however, that expire actively even at rest. Both horses and dogs recruit their abdominal muscles (specifically the transversus abdominis (TA)) at the end of the breathing cycle, generating a late expiratory (late-E) AE at rest (Gilmatin et al., 1987; Koterba et al., 1988; De Troyer et al., 1989; Koterba et al., 1995; De Troyer and Loring, 2011). Alternatively, the short-tailed opossum recruits its abdominal muscles (internal and external oblique, rectus and transversus abdominis, and pyramidalis muscles) during the first half of the expiratory phase, generating an early expiratory (early-E) AE at rest (Reilly and White, 2009; Reilly et al., 2010).

These observations led us to ask how widespread AE is under eupneic conditions in mammals, and when present, does it occur in late-E or early-E? They also raised questions about
where in the respiratory cycle AE occurs with increasing levels of respiratory drive. Hypercapnia is a respiratory stimulant that has been shown to recruit or enhance AE in a number of mammalian species (rats: Sherrey et al., 1988; Janczewski et al., 2002; Abdala et al., 2009; Taccola et al., 2007; Pagliardini et al., 2011; dogs: De Troyer et al., 1989; goats: O’Halloran et al., 1999; humans: Abe et al., 1996). We have previously hypothesized that if AE is recruited to increase tidal volume, such as during exposure to hypercapnia, then abdominal muscle contraction would occur at the end of expiration, immediately preceding inspiration allowing for a forceful contraction of the abdomen, causing an exhalation that would recruit the expiratory reserve volume (ERV; Jenkin and Milsom, 2014).

This study examined the presence of AE at rest and under progressive hypercapnia in different rodent species. The order Rodentia was chosen, as it is the largest order of mammals, composed of species displaying a wide range of sizes (from the 3-4g pygmy jerboa to the 30-90kg capybara), developmental differences (precocial vs. altricial), varied lifestyles (hibernators vs. non-hibernators) and habitats (high altitude vs. burrows) – all factors that could result in different ventilatory requirements. A ten species comparison was used to investigate which, if any, of these rodent species exhibit AE at rest, where within the breathing cycle the AE occurs at rest as well as with increasing levels of respiratory drive (hypercapnia) and how the typical I, E1 and E2 phases accommodate the presence of AE.

5.2 Methods

All experiments were performed with approval from the University of British Columbia Animal Care Committee (A13-0025), under the guidelines of the Canadian Council for Animal Care (CCAC).
5.2.1 Experimental animals

Ten different species of rodents were used: gerbils (n=2), mice (n=2), spiny rats (n=2), hamsters (n=2), ground squirrels (n=2), guinea pigs (n=2), rats (n=8), agouti (n=2), paca (n=2), and capybaras (n=2) (see Table 5.1 for body weights of the animals). All experimental trials were performed *in vivo*, and animals were unanesthetized during the duration of data collection, except for the agoutis, pacas and capybaras, which were all anesthetized with an injection of ketamine (5mg/kg), and maintained at a surgical level of anesthesia throughout the duration of the experiment.

5.2.2 Surgical procedure

Animals were anesthetized with ~2-4% isoflurane (except for agoutis, pacas, capybaras which were anesthetized with ketamine) for the surgical placement of electromyography (EMG) electrodes and impedance leads. A surgical level of anesthesia was determined when the toe pinch and corneal reflexes were abolished. An analgesic (metacam 1ml/mg) was provided subcutaneously, and subcutaneous injections of lidocaine were provided at the incision sites. In addition, the agoutis, pacas, and capybaras were tracheostomized to monitor airflow.

Coated silver wires were used to record EMG activity of select inspiratory and expiratory muscles. A small portion of the wire coating was removed at the end of each lead; this uncoated end was curved to form a hook. Coupled EMG silver wire electrodes were inserted into the diaphragm (DIA\_m) and the abdominal muscles (ABS\_m) via 21G hypodermic needles. Placement of the DIA\_m EMGs was towards the left side of the ventral surface, sliding the needles and the leads under the ribcage and advancing them cranially until they were inserted into the
diaphragm. Placement of the ABS EMGs was towards the lateral side of the lower 3rd ventral abdominal surface, with care taken to reach through all the abdominal muscles layers without perforating the abdominal viscera. EMG grounds were placed in the back muscles of all animals, except the agoutis, pacas and capybaras, where they were placed into the lower limb. In each case the needles and leads were advanced into the muscle until the hook was imbedded securely. The impedance leads were secured to shaved patches of skin on the lateral abdomen, as described by Harris (1998). The EMG and impedance leads were fed subcutaneously to an incision at the back of the neck to ensure the animals did not damage the leads during the experiment. This was not necessary for the anesthetized animals. After the successful placement of the leads, the gerbils, mice, hamsters, spiny rats, ground squirrels, guinea pig, and rats were allowed to recover from the anesthetic and were placed in an experimental chamber and exposed to air (normoxic normocapnia) for at least 20 minutes. The agoutis, pacas and capybaras remained anesthetized for the duration of the experiment. Impedance leads were connected to an impedance converter (991, Biocom Inc.), and EMG leads were connected to amplifiers (Dam50; World Precision Instruments, Inc.), filtered (0.1kHz-1kHz) and activity was sampled at 2kHz and stored on computer using a Windaq data acquisition system (D1200; DataQ Instruments) or LabChart 7 (ADI Instruments). The activity from each muscle was recorded simultaneously. The placement of the leads was verified by visual examination after each experiment.

5.2.3 Experimental procedure

Following the 20-minute exposure to air, the animals were exposed to progressive hypercapnia (0%, 2%, 4%, 6%, 8% and 10% CO₂ balanced with air, for 10 minutes at each exposure level). Gas levels were produced by mixing 100% CO₂ with air using rotameters
(60648, Cole Parmer) and analyzed with a gas analyzer (ML205 Gas Analyser, ADInstruments). For the agoutis, pacas and capybaras, the various levels of hypercapnia were administered to the animal by modifying the flow of 100% CO₂ mixed with air that passed the opening of the tracheal cannula.

5.2.4 Data analysis

5.2.4.1 Tidal volume and breathing frequency

Breathing frequency and tidal volume values were calculated for all breaths over a 1-minute period at each CO₂ exposure. When breathing was measured with impedance, the frequency of the impedance trace cycle was used to determine breathing frequency, while the amplitude in the impedance trace deflection was used as a correlate of tidal volume. When breathing was measured with airflow (the agoutis, pacas and capybaras), breathing frequency was determined by counting the number of breaths in a 1-minute period. The pressure transducer were calibrated prior to the experiment and used to record airflow throughout the duration of the experiment. Inspiratory and expiratory volumes were integrated independently and summed to determine tidal volume for each breath. The amplitude of the tidal volume trace over one minute was averaged to represent the tidal volume for each experimental condition.

5.2.4.2 EMG trace analysis

The DC offset of the EMG signals was removed, the raw traces were rectified, and the absolute integral was calculated and smoothed (50 msec) for analysis (LabChart7, ADInstruments; as described by Lemes and Zoccal, 2014). The start and end of the diaphragm and abdominal muscle activity associated with each breath were recorded. For the DIAₘ, this
was done to quantify the duration of the I phase (the contraction of the DIA$_m$ during the inspiration portion of the impedance trace), the E1 phase (the prolonged contraction of DIA$_m$ during the expiration portion of the impedance trace), and the E2 phase (the remaining expiratory portion of the impedance trace that had no DIA$_m$ activity). For the ABS$_m$, the durations of ABS$_m$ activity were calculated along with the offset of the starting time of the ABS$_m$ activity relative to the starting time of the DIA$_m$ muscle activity.

T$_I$ was measured as the time from the start of the DIA$_m$ activity until the transition between inspiration and expiration on the airflow or impedance trace. T$_{E1}$ was measured as the time period during which DIA$_m$ activity continued into the expiratory phase. T$_{E2}$ was the period of no muscle contraction during expiration and T$_{AE}$ was measured as the time during which phasic ABS$_m$ activity was present during the expiratory phase. The total time from the start of the DIA$_m$ activity from one breath to the start of the DIA$_m$ activity to the next breath was determined as T$_{TOT}$ (Figure 5.1). This was done for 10 consecutive breaths and averaged for each animal at each CO$_2$ level. The duty cycles for T$_I$, T$_{E1}$ and T$_{AE}$ (T$_I$/T$_{TOT}$, T$_{E1}$/T$_{TOT}$, and T$_{AE}$/T$_{TOT}$, respectively) were calculated for 10 consecutive breaths and averaged for each CO$_2$ level.

5.2.5 Statistical analysis

All values represent means ± standard error of the mean. Variability between the data of the 10 rodents species was evaluated with the interquartile range (IQR). Regressions were calculated using SigmaStat.

5.3 Results

The average breathing frequencies and respiratory phase durations for each species under
normoxic normocapnic (eupneic) conditions are presented in Table 5.1. Active expiration was never consistently observed in these rodent species at rest. However, gerbils and mice both exhibited occasional AE under eupneic conditions (Figure 5.2). These abdominal muscle contractions occurred every 12±1.36 breaths in mice and every 20±5.73 breaths in gerbils. Thus the general breathing cycle of all species under eupneic conditions was composed of three phases: I, E1, and E2. The inspiratory duty cycle (T_I/T_{TOT}), the fraction of the breath spent inspiring, ranged from 0.40 to 0.68, (IQR=0.12) (5.3A,B). There is a weak correlation between the inspiratory duty cycle and breathing frequency (R^2=0.223) (Figure 5.4). Of the total expiratory time, E1 generally contributed less than E2; T_{E1}/T_E ranged from 0.06 to 0.54 (IQR=0.14) (Figure 5.5A,B)). There was no correlation between the expiratory braking duty cycle and breathing frequency (R^2=1.93 x 10^-4) (Figure 5.6A). While there was a trend for the E2 phase to become prolonged in the three largest species, with the slowest breathing frequencies, the trend, if anything, was opposite in other rodents. However, there is a weak negative correlation between the E2 duty cycle (E_2/T_{TOT}) and breathing frequency (R^2=0.173) (Figure 5.6B).

5.3.1 Breathing frequency and tidal volume changes with progressive hypercapnia

Exposure to progressive hypercapnia resulted in an overall increase in tidal volume (V_T) for all species, except for hamsters (Figure 5.7). Exposure to progressive hypercapnia also resulted in an initial increase in breathing frequency (f_R) followed by a decrease in f_R at more severe levels of hypercapnia in most species (hamsters, ground squirrels, rats, guinea pigs and pacas). This secondary decline in f_R was not seen in spiny rats and agoutis. Also, mice and gerbils exhibited a consistent decrease in f_R, and capybaras exhibited no change in f_R (capybaras).
Thus the extent of the decrease in $V_T$ and increase in $f_R$ varied across species (Figure 5.7).

### 5.3.2 Inspiratory and expiratory phase changes with progressive hypercapnia

The duration of the inspiratory phase ($T_I$) exhibited little consistent change with progressive hypercapnia (Figure 5.8). The only exceptions were gerbils, which had a substantial increase in $T_I$ at severe levels of hypercapnia, and the agoutis, which exhibited a decrease in $T_I$ at severe levels of hypercapnia. As $T_I$ did not change while $V_T$ increased substantially with progressive hypercapnia, estimates of mean inspiratory flow, represented as $V_T/T_I$ also increased substantially with progressive hypercapnia in all species except the mouse (Figure 5.9).

The inspiratory duty cycle ($T_I/T_{TOT}$) fluctuated with progressive hypercapnia, typically decreasing with the appearance of AE, with the exceptions of the ground squirrels, rats and capybaras (Figure 5.10). As a result $T_E/T_{TOT}$ typically increased with the appearance of AE compared to normocapnic conditions. The expiratory braking duty cycle ($T_{EI}/T_{TOT}$) consistently decreased with progressive hypercapnia with the appearance of AE, with the exception of gerbils and pacas, which exhibited an increase in $T_{EI}/T_{TOT}$ and the capybaras and spiny rats, which exhibited no change in $T_{EI}/T_{TOT}$. Interestingly, under normocapnic conditions, there was great consistency in $T_{EI}/T_{TOT}$ among species, ranging from 0.03 to 0.27 (IQR=0.09; Figure 5.11). $T_{AE}/T_{TOT}$, the expiratory duty cycle, remained fairly constant with increasing levels of hypercapnia, ranging from 0.11 to 0.44 (IQR= 0.17; Figure 5.11). Mice and capybaras were the exception with $T_{AE}/T_{TOT}$ decreasing at the highest levels of hypercapnia (Figure 5.10).

### 5.3.3 Active expiation occurs under hypercapnic conditions

While no consistent AE was observed at rest, hypercapnia eventually produced an AE in
all rodent species. However, the level of CO₂ required to recruit AE varied among species. Hamsters, ground squirrels and guinea pigs recruited AE at 6% CO₂, gerbils, mice, rats and capybaras recruited AE at 8% CO₂, and spiny rats, agoutis and pacas did not recruit AE until 10% CO₂. The appearance of AE typically occurred concurrently with a substantial increase in Vₜ for all species (see Figure 5.7).

The placement of AE within the breathing cycle also varied among species. The majority of the species (gerbil, mice, hamster, spiny rat, rat, pacas, and capybaras) exhibited a late expiratory (late-E) AE, generating a four-phase breathing cycle that consisted of I, E₁, E₂ and AE (Figure 5.12A). In ground squirrels and guinea pigs, AE occupied the entire expiratory phase, generating a two-phase breathing cycle of I followed by AE (Figure 5.12B). Agoutis were the exception, exhibiting an early expiratory (early-E) AE, generating a three-phase breathing cycle that consisted of I, AE, and E₂ (Figure 5.12C). Note that for the guinea pigs and agouti, expiratory braking also occurred even though AE commenced immediately following inspiration. Also note that with the exception of the agouti, once recruited, ABSₘ activity persisted into the I phase.

5.4 Discussion

5.4.1 Rodents do not exhibit AE at rest

Based on the ten species examined in this study, most rodents do not appear to exhibit consistent active expiration (AE) under eupneic conditions. However, gerbils and mice did recruit AE occasionally (on average AE was recruited every 12 breaths and every 20 breaths in gerbils and mice, respectively). Characterization of these breaths has proven challenging. It has been suggested that these breaths represent sighs. Based on spirometry and impedance
plethysmography data, sighs typically show a large inspiration, which recruits the inspiratory reserve volume (IRV), rather than a large expiration, which would recruit the expiratory reserve volume (ERV) (Li et al., 1977). Based on airflow traces, sighs exhibit an enhanced inspiration, composed of two phases: a eupneic breath followed by an augmented breath. This enhanced inspiration can be followed by an enhanced expiration (Nguyen et al., 2012). Based on these characteristics, it would appear as though the breaths demonstrated by mice and gerbils should not be considered sighs. Firstly, the impedance traces of mice and gerbils do not show an enhanced inspiration typical of a sigh (Figure 5.2). In addition, the drifting upward slope of the gerbil and mouse impedance curves after these AE breaths suggests the animals are re-inflating the lungs, returning to their normal end-expiratory lung volume. The elevated end-expiratory lung volume is likely due to the enhanced breathing frequencies of these species leaving insufficient time for passive expiration. Potentially, this build up of the end-expiratory volume allows for elastic recoil forces to balance inspiratory volume with expiratory volume, generating a dynamic FRC. This AE is required to produce a forceful expiration as a means to “reset” their lung volumes, only to have the build up in the end-expiratory volume occur again.

5.4.2 All rodents exhibit a three phase breathing cycle at rest

It has been predicted and calculated that $T_E/T_{TOT}$ is an interspecific constant of roughly 65% for a number of awake, resting mammalian species (Boggs and Tenney, 1984). In our study, this ratio of $T_E/T_{TOT}$ ranged from 40% to 64% with a mean of roughly 50%. Table 5.2 provides the values of $T_E/T_{TOT}$ for mice, hamsters and rats calculated by Boggs ad Tenney (1984) and recorded in the present study. Despite the difference in the percentage of $T_E/T_{TOT}$ between Boggs and Tenney (1984) and our study, there was little difference in the calculated interquartile ranges
(IQR was 0.08 and 0.12 for Boggs and Tenney and the present study, respectively). The similarity in the interquartile range for $T_E/T_{TOT}$ suggests a similar interspecific relationship of $T_E/T_{TOT}$ across species.

As best we know, there are no studies reporting whether there is also an interspecific constant for the post-inspiratory diaphragmatic activity that slows expiration (expiratory braking) or for the active expiratory activity of the abdominal muscles that enhances expiration (when present). There appears to be substantial interspecies variation in the duration of expiratory braking (E1) exhibited by mammals (Boggs, 1992), as expiratory braking is often considered to be more important in neonates of a number of species (humans: Mortola et al., 1982; dogs: England et al., 1985; and sheep: Harding et al., 1980) and in adults of species with highly compliant chest walls (Vinegar et al., 1979; Remmers and Bartlett, 1979; Hazari and Farraj, 2015; LoMauro and Aliverti, 2016). Our study found little interspecies variability in $T_{E1}/T_{TOT}$ with values ranging from 0.03 to 0.27 (IQR = 0.08) in adult rodents. These similar expiratory braking duty cycles may be due to similarities in chest wall mechanics of adult rodents or similarities in the neural control of the diaphragmatic post-inspiratory activity (Richter et al., 1987; Subramanian and Holstege, 2011). AE was never present consistently under eupneic conditions, however, when AE was fully established in all species (10% CO$_2$), there was not a substantial range of $T_{AE}/T_{TOT}$ with values ranging from 0.11 to 0.44 (IQR = 0.17) among these rodent species. Again, this may be due to similarities in pulmonary mechanics or similarities in the activation of the late-E neural activity.

5.4.3 The effect of progressive hypercapnia on the breathing cycle

Progressive hypercapnia produced increases in tidal volume ($V_T$) in all species and
modest increases in breathing frequency in most (Figure 4). The changes in breathing frequency were due to changes in some combination of $T_I$, $T_{E1}$, $T_{E2}$, and $T_{AE}$ at higher levels of respiratory drive. With some exceptions (gerbils, hamsters, spiny rats, and agoutis), there were few differences when comparing $T_I$ at 0% CO$_2$ and 10% CO$_2$. Also with few exceptions, $T_{E1}$ and $T_{E2}$ decreased from 0% inspired CO$_2$ to 10% CO$_2$. These results are similar to the work of Smith et al., (1989), which found that $T_I$ changed minimally between control (air) and hypercapnic (6.5% CO$_2$) conditions (~0.5s decrease), while $T_{E1}$ and $T_{E2}$ decreased substantially (~0.12s and ~0.98s, respectively). While both $T_{E1}$ and $T_{E2}$ decreased in our study, the overall $T_E$ did not decrease due to the arrival of $T_{AE}$. The arrival of AE at more severe levels of hypercapnia appears to be the reason for the increase in $T_{TOT}$ and the reduction in breathing frequency at the higher levels of inspired CO$_2$.

The inspiratory and expiratory duty cycles did fluctuate with progressive hypercapnia, with $T_E/T_{TOT}$ decreasing and $T_I/T_{TOT}$ increasing from 0% CO$_2$ to 6% CO$_2$. However, as the levels of hypercapnia became more severe (8%-10% CO$_2$), $T_E/T_{TOT}$ began to increase while $T_I/T_{TOT}$ decreased. This suggests that at high levels of hypercapnia, expiration becomes the more predominant phase of the breathing cycle. Within expiration, the expiratory braking duty cycle ($T_{E1}/T_{TOT}$) decreased with progressive hypercapnia. While there was a reduction in expiratory braking by the DIA$_m$, there may be an increase in expiratory braking by other laryngeal muscles. In adult cats, hypercapnia reduces diaphragmatic expiratory braking, but enhances braking by the laryngeal muscles (Bartlett et al., 1973).
5.4.4 Hypercapnia recruited active expiration in all rodents

Elevated levels of hypercapnia consistently recruited AE in the 10 rodent species studied. The level of CO$_2$ required to recruit AE, however, varied across species. 8% CO$_2$ was sufficient to recruit expiratory abdominal muscle activity with every breath in rats in situ (Abdala et al., 2009). Current models of the neural circuitry that powers breathing suggest that the recruitment of late expiratory (late-E) abdominal nerve activity arises from excitation of chemosensitive cells in the RTN/pFRG (Guyenet et al., 2009; Molkov et al., 2014), highlighting the importance of CO$_2$ in generating AE. 8% CO$_2$ was the most common level of CO$_2$ required to recruit AE in rodents in the present study (rats, mice, gerbils, paca and capybara), suggesting that CO$_2$ chemosensitivity of the RTN/pFRG is similar across species. However, there are some differences in CO$_2$ sensitivity as hamsters, guinea pigs and ground squirrels recruited AE at 6% CO$_2$ and spiny rats and agoutis not until 10% CO$_2$.

With the earlier appearance of AE, there is often an earlier increase in $V_T$. In previous studies, hamsters showed a greater increase in $V_T$ compared to rats at 5% and 10% inspired CO$_2$ (Walker et al., 1985). Guinea pigs have also been shown to significantly increase $V_T$ at 5% CO$_2$ (Blake and Banchero, 1985). Webb and Milsom (1994) found that golden-mantled ground squirrels increased overall ventilation at 6% CO$_2$ due to a substantial increase in $V_T$ with little change to $f_R$. While these results are from a different species of ground squirrel (Callospermophilus lateralis compared to Ictidomys tridecemlineatus), they do highlight a heightened CO$_2$ sensitivity to hypercapnia similar to what was observed in the present study. Based on these observations, we propose that the recruitment of AE is critical to produce significant increases in tidal volume. Barros et al., (1998) found that spiny rats (P. yonenagae and P. inherigni) have a stronger $V_T$ response to CO$_2$ than rats. This is inconsistent with our data,
as we found that spiny rats showed very modest increases in $V_T$ and required exposure to 10% $\text{CO}_2$ to recruit AE.

The way in which AE was recruited varied among species. The majority of rodents (gerbils, mice, hamsters, spiny rats, rats, pacas and capybaras) exhibited a four-phase breathing cycle with late-E active expiration producing a breathing cycle composed of: I, E1, E2 and AE. A number of studies that have investigated the appearance of AE across mammals with increasing respiratory drive also found that AE appears at the end of the expiratory phase (late-E) (humans: Abe et al., 1996; rats: Sherrey et al., 1988; Janczewski et al., 2002; Abdala et al., 2009; Taccola et al., 2007; dogs: De Troyer et al., 1989; goats: O’Halloran et al., 1999). Typically, it has been shown that as respiratory drive increases further, this late-E AE extends further into E2 (Gautier et al., 1973; Sherrey et al., 1988; De Troyer et al., 1989). This is also the case in the present study. In pacas, E2 was almost eliminated and in ground squirrels and guinea pigs it was totally eliminated. Interestingly, when AE is present in a late-E phase, E1 (i.e. some $\text{DIA}_m$ activity during early expiration) always seems to be conserved in the breathing cycle (Sherrey et al., 1988).

Agoutis exhibited a three-phase breathing cycle with an early-E AE (producing a breathing cycle composed of: I, AE and E2). This is similar to the breathing cycle of the short-tailed opossums under eupneic conditions (Reilly and White, 2009). The reason for this early-E AE is still unclear. It has been suggested that some animals require an early AE to match a high-speed ventilatory system (Reilly and White, 2009). As was hypothesized in Chapter 1, an early-E AE may help animals with highly compliant chest walls to exhale at a faster speed to counteract a passive expiratory time constant. Interestingly, the rodents from our study with the highest breathing frequencies (mice and gerbils) did not exhibit an early-E AE, suggesting that
high breathing frequencies are not dependent on an early-E AE. Again, without this early-E AE, gerbils and mice appear to be hyper-inflating their lungs, potentially generating an increase in elastic recoil, which would balance inspiration and expiration. It is still possible that the time required for the passive collapse of the respiratory system of agoutis, however, is sufficiently long that this species is dependent on an early-E AE to enhance expiratory airflow. Evident from Figure 5.12, there is a concurrent expiratory braking (E1 DIA_m activity) and AE activity (early-E ABS_m activity). The E1 DIA_m activity of the agouti, however, was substantially smaller than that of any other rodent (T_E1/T_E was consistently under 10%, see Figure 6), suggesting that it does not contribute substantially to the overall breathing cycle. It is not clear what the purpose is of the second expiratory phase (E2) after an early-E AE (during E1). Based on the increase in tidal volume, it would appear as though the animals are recruiting their ERV during this time. The maintained airflow during expiration after this abdominal muscle activity (Figure 5.12C) suggests this AE helps in speeding up airflow at the beginning of the expiratory phase. The continued expiratory airflow after this abdominal muscle activity finished might be due to some other expiratory muscle activity not recorded in this study.

We hypothesized previously that an early-E AE may eliminate the expiratory braking phase and help counteract airway resistance to speed up expiration (Chapter 1) and that a late-E AE would help to recruit the ERV and allow for an increase in V_T (Chapter 1). Based on the substantial increase in V_T seen in all species in this study, AE appears to help increase V_T by recruiting the expiratory reserve volume regardless of its placement.

5.4.5 Conclusion

The results from this study highlight more similarities than differences in respiratory
muscle recruitment under progressive hypercapnia across rodent species. While hypercapnia recruits AE in all rodents, AE is recruited at different CO$_2$ levels. In addition, it appears as though the majority of species recruit AE in a similar manner, generating phasic $ABS_m$ activity at the end of the breathing cycle. The only true exception were the agoutis, which recruited AE at the beginning of the expiratory phase. When the different respiratory muscles are recruited by these rodent species may be a function both of the mechanics of the respiratory system (e.g., chest wall compliance) and/or CO$_2$ chemosensitivity. Future studies will examine if any differences in respiratory mechanics could account for the different recruitment patterns demonstrated by these species.
Figure 5.1 Representative traces of breathing and the respiratory muscles. Trace of breathing (measured with impedance, upward inflection represents an inspiration), the raw and integrated diaphragm ($\text{DIA}_m$) and abdominal muscles ($\text{ABS}_m$) are presented. The vertical dotted lines represent the four potential phases of the breathing cycle (inspiration, I, blue bars; expiratory braking, E1, green bar; second expiratory phase, E2 yellow bar, and an active expiration, AE, red bar.)
Figure 5.2 Representative trace of the occasional active expiration exhibited by gerbils and mice. The breathing trace (measured with impedance, upward inflection represents an inspiration) suggests that with each phasic abdominal burst ($\text{ABS}_m$) there is substantially emptying of the lungs, followed by a progressive hyperinflation of the lungs.
Figure 5.3 Comparing the inspiratory duration (T\textsubscript{I}) to the total expiratory phase duration (T\textsubscript{E}) among a number of rodent species under eupneic conditions. A) The absolute duration (seconds) of the inspiratory time (T\textsubscript{I}, blue bars) and the total expiratory time (T\textsubscript{E}, grey bars) are presented for each species under eupneic conditions. B) The duration of the inspiratory phase (T\textsubscript{I}, blue bars) and of the expiratory phase (T\textsubscript{E}, grey bars) as a ratio of the total breathing cycle time (T\textsubscript{TOT}) under eupneic conditions. Bars represent mean values ± S.E.M.
Figure 5.4 Relationship between breathing frequency (min⁻¹) and the inspiratory duty cycle (Tᵢ/Tₜₒₜ). Line represents a positive linear regression trend (R²=0.223). Data points represent mean values ± S.E.M.
Figure 5.5 Comparing the expiratory braking phase duration ($T_{E1}$) and the second expiratory phase duration ($T_{E2}$) among a number of rodent species under eupneic conditions. A) The absolute duration (seconds) of the expiratory braking time ($T_{E1}$, green bars) and the second expiratory phase ($T_{E2}$, yellow bars) are presented for each species under eupneic conditions. B) Ratio of $T_{E1}$ (green bars) and $T_{E2}$ (yellow bars) as a ratio of the total expiratory time ($T_E$) for each rodent species under eupneic conditions. Bars represent mean values ± S.E.M.
Figure 5.6 Relationship between A) breathing frequency (min\(^{-1}\)) and expiratory braking (T_{E1}/T_{TOT}) and between B) breathing frequency (min\(^{-1}\)) and the second expiratory phase duty cycle (T_{E2}/T_{TOT}). Data points represent mean values ± S.E.M.
Figure 5.7 Effect of progressive hypercapnia on the tidal volume and breathing frequency of 10 rodent species. Tidal volume (purple bars) and breathing frequency (green bars) of 10 rodent species when exposed to 0%, 2%, 4%, 6%, 8% and 10% progressive CO₂ (Agouti, Paca and Capybara did not receive 2% and 4% CO₂). The red hatched bars represent the CO₂ levels at which active expiration is present in each species. Bars represent mean values ± S.E.M.
Figure 5.8  Effect of progressive hypercapnia on the duration of the breathing cycle phases of 10 rodent species. The inspiratory phase (I, blue bars), expiratory braking phase (E1, green bars), secondary expiratory phase (E2, yellow bars) of the diaphragm and active expiratory phase (AE, red bars) of the abdominal muscles are presented for 10 rodent species exposed to 0%, 2%, 4%, 6%, 8% and 10% progressive CO$_2$ (Agouti, Paca and Capybara did not receive 2% and 4% CO$_2$). Bars represent mean values ± S.E.M.
Figure 5.9 Effect of progressive hypercapnia on $V_T/T_1$ of 10 rodent species. $V_T/T_1$ is an estimate of airflow for the 10 rodent species exposed to 0%, 2%, 4%, 6%, 8% and 10% progressive CO$_2$ (Agouti, Paca and Capybara did not receive 2% and 4% CO$_2$). The red hatched bars represent the CO$_2$ levels at which active expiration is present in each species. Bars represent mean values ± S.E.M.
Figure 5.10 Effect of progressive hypercapnia on the duty cycles of the breathing phases of 10 rodent species. The inspiratory phase (I, blue bars), expiratory braking phase (E1, green bars), secondary expiratory phase (E2, yellow bars) of the diaphragm and active expiratory phase (AE, red bars) of the abdominal muscles are presented as a ratio of the total breathing cycle time ($T_{TOT}$) for 10 rodent species. Animals were exposed to 0%, 2%, 4%, 6%, 8% and 10% progressive CO$_2$ (Agouti, Paca and Capybara did not receive 2% and 4% CO$_2$).
Figure 5.11 Average duty cycles for expiration ($T_E$), expiratory braking ($T_{E1}$) and active expiration ($T_{AE}$) exhibited by 10 rodent species. Values for $T_E$ and $T_{E1}$ were taken from eupneic conditions, while values for $T_{AE}$ was taken when active expiration was fully established for all species (10% CO$_2$).
Figure 5.12 Representative traces of the different breathing patterns generated when active expiration is present. A) Representation of a four-phase breathing cycle. The vertical dotted lines identify the inspiratory phase (I, blue bar), an expiratory braking phase (E1, green bar), an expiratory pause (E2, yellow bar) and active expiration (AE, red bar). Breathing is measured with impedance, and an upward deflection represents inspiration. B) Representation of a two-phase breathing cycle. The vertical dotted lines identify the inspiratory phase (I, blue bar) and active expiration (AE, red bar). Breathing is measured with impedance, and an upward deflection represents inspiration. C) A representation of a three-phase breathing cycle. The vertical dotted lines identify the inspiratory phase (I, blue bar), active expiration (AE, red bar) and an expiratory pause (E2, yellow bar). Breathing is represented with airflow, and a positive movement represents inspiration.
Table 5.1 Breathing parameters of 10 rodent species under eupneic conditions. The number of individuals (n), mean mass (kg), breathing frequency (fR), calculated tidal volume (V_T) and respiratory phase durations for each species in normoxic normocapnic conditions.

<table>
<thead>
<tr>
<th>Species</th>
<th>n</th>
<th>Mass (kg)</th>
<th>f_R (min⁻¹)</th>
<th>V_T (ml)</th>
<th>T₁ (sec)</th>
<th>Tₑ₁ (sec)</th>
<th>Tₑ₂ (sec)</th>
<th>T_TOT (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gerbil</td>
<td>2</td>
<td>0.05 ± 0.002</td>
<td>232.50 ± 18.98</td>
<td>0.33 ± 0.03*</td>
<td>0.15 ± 0.001</td>
<td>0.029 ± 0.002</td>
<td>0.073 ± 0.006</td>
<td>0.26 ± 0.012</td>
</tr>
<tr>
<td>Mouse</td>
<td>2</td>
<td>0.05 ± 0.003</td>
<td>128.75 ± 27.49</td>
<td>0.40 ± 0.05*</td>
<td>0.27 ± 0.057</td>
<td>0.11 ± 0.015</td>
<td>0.18 ± 0.003</td>
<td>0.58 ± 0.074</td>
</tr>
<tr>
<td>Spiny Rat</td>
<td>2</td>
<td>0.09 ± 0.000</td>
<td>123.25 ± 8.75</td>
<td>0.83 ± 0.01*</td>
<td>0.28 ± 0.037</td>
<td>0.077 ± 0.008</td>
<td>0.13 ± 0.004</td>
<td>0.49 ± 0.040</td>
</tr>
<tr>
<td>Hamster</td>
<td>2</td>
<td>0.13 ± 0.002</td>
<td>105.75 ± 15.52</td>
<td>1.07 ± 0.00</td>
<td>0.27 ± 0.023</td>
<td>0.079 ± 0.028</td>
<td>0.26 ± 0.048</td>
<td>0.61 ± 0.099</td>
</tr>
<tr>
<td>Ground Squirrel</td>
<td>2</td>
<td>0.32 ± 0.001</td>
<td>109.00 ± 10.29</td>
<td>2.32 ± 0.23*</td>
<td>0.25 ± 0.011</td>
<td>0.081 ± 0.010</td>
<td>0.18 ± 0.031</td>
<td>0.52 ± 0.032</td>
</tr>
<tr>
<td>Rat</td>
<td>8</td>
<td>0.42 ± 0.02</td>
<td>81.19 ± 2.6875</td>
<td>3.13 ± 0.12*</td>
<td>0.31 ± 0.036</td>
<td>0.18 ± 0.023</td>
<td>0.21 ± 0.024</td>
<td>0.70 ± 0.034</td>
</tr>
<tr>
<td>Guinea Pig</td>
<td>2</td>
<td>0.46 ± 0.14</td>
<td>99.50 ± 12.33</td>
<td>3.41 ± 1.05*</td>
<td>0.27 ± 0.008</td>
<td>0.11 ± 0.008</td>
<td>0.20 ± 0.005</td>
<td>0.58 ± 0.020</td>
</tr>
<tr>
<td>Agouti</td>
<td>2</td>
<td>3.10 ± 0.10</td>
<td>92.00 ± 5.96</td>
<td>4.68 ± 0.46</td>
<td>0.40 ± 0.022</td>
<td>0.022 ± 0.005</td>
<td>0.24 ± 0.013</td>
<td>0.64 ± 0.022</td>
</tr>
<tr>
<td>Paca</td>
<td>2</td>
<td>8.70 ± 0.20</td>
<td>97.50 ± 7.50</td>
<td>9.08 ± 0.02</td>
<td>0.44 ± 0.12</td>
<td>0.034 ± 0.021</td>
<td>0.17 ± 0.024</td>
<td>0.64 ± 0.119</td>
</tr>
<tr>
<td>Capybara</td>
<td>2</td>
<td>31.40 ± 0.60</td>
<td>35.33 ± 7.42</td>
<td>18.97 ± 0.61</td>
<td>0.59 ± 0.008</td>
<td>0.25 ± 0.094</td>
<td>1.06 ± 0.30</td>
<td>1.91 ± 0.38</td>
</tr>
</tbody>
</table>

* V_T calculated using equation from Stahl, 1967 based on mass.
Table 5.2 Comparison of $T_E/T_{TOT}$ between present study and Boggs and Tenney (1984).

Calculated values for $T_E/T_{TOT}$ as presented by Boggs and Tenney (1984) and the present study for mice, hamsters and rats.

<table>
<thead>
<tr>
<th></th>
<th>Boggs and Tenney, 1984</th>
<th>Present Study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ratio of $T_E/T_{TOT}$</td>
<td>Ratio of $T_E/T_{TOT}$</td>
</tr>
<tr>
<td>Mice</td>
<td>0.729</td>
<td>0.483</td>
</tr>
<tr>
<td>Hamsters</td>
<td>0.639</td>
<td>0.428</td>
</tr>
<tr>
<td>Rats</td>
<td>0.658</td>
<td>0.549</td>
</tr>
</tbody>
</table>
Chapter 6: Respiratory muscle recruitment under hypoxic conditions: a comparative analysis in rodents

6.1 Introduction

At rest, the mammalian breathing cycle is typically composed of three phases. The breathing cycle starts with an active inspiration (I), dependent on the contraction of the inspiratory muscle, such as the diaphragm and the external intercostal muscles, to draw air into the lungs. Expiration is divided into two phases. The first expiratory phase (E1) is characterized by a prolonged contraction of the inspiratory muscles that brake the rate at which air is exhaled from the lungs (E1) and the second expiratory phase (E2) is characterized by passive expiration and, depending on the rate of breathing, a pause with no muscle contraction and no airflow (Richter, 1996; Feldman and McCrimmon, 2003). Normally under resting conditions, inspiration is active, and expiration is passive.

The hypoxic ventilatory response in rodents is characterized by a substantial increase in breathing frequency, with little change in tidal volume ($V_T$; Cragg and Drysdale, 1983). To increase breathing frequency, the total breathing cycle time ($T_{TOT}$) must decrease. This decrease in $T_{TOT}$ is typically due to a more pronounced decrease in expiratory time ($T_E$) than in inspiratory time ($T_I$) (cats: Lovering et al., 2003; dogs: Smith et al., 1989; rats: Saiki et al., 2007). However, few studies have distinguished whether this decrease in $T_E$ is due to a decrease in $T_{E1}$, $T_{E2}$ or a combination of the two. Reducing the expiratory pause (seen during $T_{E2}$) would shorten the breath length ($T_{TOT}$) and reducing expiratory braking ($T_{E1}$) would enhance expiratory airflow and shorten $T_{TOT}$ further. If breathing frequency increases to the point that $T_E$ must be reduced past the minimum time required for passive deflation of the respiratory system, then an active
expiration would be required to adequately empty the lungs.

In the present study, we investigated the extent to which changes in $T_1$, $T_{E1}$ and $T_{E2}$ contribute to the reduction in $T_{TOT}$ as breathing frequency increases in hypoxia. It was hypothesized that $T_{E2}$ would be reduced first, that $T_{E1}$ would subsequently disappear, that the $T_1/T_E$ ratio would increase and that if breathing frequency increased sufficiently, then a forceful contraction of the abdominal muscles during expiration would be recruited to enhance expiratory airflow and reduce $T_E$ past the time required for passive elastic recoil of the respiratory system.

6.2 Methods

All experiments were performed with approval from the University of British Columbia Animal Care Committee (A13-0025), under the guidelines of the Canadian Council for Animal Care (CCAC).

6.2.1 Experimental animals

Seven different species of rodents were used: gerbils (n=2), mice (n=2), spiny rats (n=2), hamsters (n=2), ground squirrels (n=2), rats (n=8), and guinea pigs (n=2). See Table 5.1 for body weights for all animals and eupneic breathing parameters. All experimental trials were performed in vivo, and animals were unanesthetized during the duration of data collection.

6.2.2 Surgical procedure

Animals were anesthetized with ~2-4% isoflurane for the surgical placement of the electromyography (EMG) and impedance leads. A surgical level of anesthesia was determined when the toe pinch and corneal reflex were abolished. An overall analgesic (metacam 1ml/mg)
was provided subcutaneously, and subcutaneous injections of lidocaine were provided at the incision sites.

6.2.3 EMG lead placement

Coated silver wires were used to record EMG activity of select inspiratory and expiratory muscles. A small portion of the wire coating was removed at the end of each lead; this uncoated end was curved to form a hook. Coupled EMG silver wire electrodes were inserted into the diaphragm (DIA$_m$) and the abdominal muscles (ABS$_m$) via 21G hypodermic needles. Placement of the DIA$_m$ EMGs was towards the left side of the ventral surface, sliding the needles and the leads under the ribcage and advancing them cranially until they were inserted into the diaphragm. Placement of the ABS$_m$ EMGs was towards the lateral side of the lower 3$^{rd}$ ventral abdominal surface, with care taken to reach through all the abdominal muscles layers without perforating the abdominal viscera. EMG grounds were placed in the back muscles of all animals. This was done by advancing the needles and leads into the muscle until the hook was imbedded securely. The impedance leads were secured to shaved patches of skin on the lateral abdomen, as described by Harris (1998). The EMG and impedance leads were fed subcutaneously to an incision at the back of the neck to ensure the animals did not damage the leads during the experiment. After the successful placement of the leads, the gerbils, mice, hamsters, spiny rats, ground squirrels, guinea pig, and rats were allowed to recover from the anesthetic and were placed in an experimental chamber and exposed to air (normoxic normocapnia) for at least 20 minutes. Impedance leads were connected to an impedance converter (991; Biocom Inc.), and EMG leads were connected to amplifiers (Dam50; World Precision Instruments, Inc.), filtered (0.1kHz-1kHz) and activity was sampled at 2kHz and stored on computer using a Windaq data
acquisition system (D1200; DataQ Instruments) or LabChart 7 (ADInstruments). The activity from each muscle was recorded simultaneously. The placement of the leads was verified by visual examination after each experiment.

### 6.2.4 Hypoxic exposure

Following the 20-minute exposure to air, the animals were exposed to progressive hypoxia (21%, 19%, 17%, 15%, 13%, 11% and 9% O\textsubscript{2} balanced with nitrogen), for 10 minutes at each exposure level. The different levels of hypoxia were established by mixing 100% N\textsubscript{2} with air using rotameters (Cole Parmer, 60648). The levels were confirmed with a gas analyzer (ADInstruments, ML205).

### 6.2.5 Data analysis

#### 6.2.5.1 Measuring tidal volume and breathing frequency

For each animal, breathing frequencies and tidal volumes were calculated for 1-minute at each O\textsubscript{2} level. Respiratory variables were measured from impedance traces; the amplitude of the impedance trace deflection was used as a correlate of tidal volume and the frequency of the impedance trace cycle was used to determine breathing frequency.

#### 6.2.5.2 EMG analysis

The DC offset of the EMG signals was removed, the raw traces were rectified, and the absolute integral was calculated and smoothed (50 msec) for analysis (LabChart7, ADInstruments; as described by Lemes and Zoccal, 2014). The only activity recorded from the ABS\textsubscript{m} was low levels of tonic activity and this activity was not analyzed. There were no bursts of
activity in this muscle group and no sign of active expiration at any level of inspired O_2 in any species. The time points for the start and end of the diaphragm activity were recorded. This was done to quantify the duration of the I phase (the contraction of the DIA_m during the inspiration portion of the impedance trace), the E1 phase (the prolonged contraction of DIA_m during the expiration portion of the impedance trace), and the E2 phase (the remaining expiratory portion of the impedance trace that had no DIA_m activity; Figure 5.1). The amount of DIA_m activity was determined using the integral relative to baseline function in LabChart7 for ten consecutive breaths at each level of inspired O_2.

T_I was measured as the time from the start of the diaphragm activity until the transition between inspiration and expiration on the impedance trace. T_{E1} was measured as the remaining diaphragm activity into the expiratory phase and T_{E2} was the period of no muscle contraction during expiration. The total time from the start of diaphragm activity from one breath to the start of the diaphragm activity of the next breath was determined as T_{TOT}. This was also done for 10 consecutive breaths and averaged for each animal at each O_2 level. The duty cycles for T_I, T_{E1} and T_{E2} and the V_T/T_I (representing mean inspiratory flow) and I/E (representing the inspiratory to expiratory time ratio) were calculated for each breath and averaged for each O_2 level.

6.2.6 Statistical analysis

All values represent means ± standard error of the mean.

6.3 Results

6.3.1 Effect of progressive hypoxia on tidal volume and breathing frequency

Exposure to progressive hypoxia resulted in a progressive increase in breathing frequency
(f_R) in rats and guinea pigs. In mice, hamsters, and ground squirrels there was an initial increase in f_R followed by a decrease at more severe levels of hypoxia. Alternatively, the gerbils exhibited very little change in f_R, and spiny rats exhibited an overall decrease in f_R. Exposure to progressive hypoxia resulted in an overall decrease in tidal volume (V_T) for all species, with the exception of hamsters, in which V_T increased until 11% O_2 and then decreased (Figure 6.1). The extent of the changes in V_T and f_R varied across species (Figure 6.1). Progressive hypoxia never recruited AE, even at the highest level of respiratory drive (9% inspired O_2).

The decreases in total breathing cycle time (T_TOT) that accompanied the increases in f_R reached a minimum at different levels of inspired O_2 in different species (see hatched bars in Figure 6.2). The overall increases in f_R and reductions in T_TOT were surprisingly small. While some species demonstrated a progressive decrease in T_TOT (i.e. rats), the rodents with the highest resting breathing frequency (i.e. gerbils) demonstrated little change in T_TOT. Overall, there was no consistent pattern in how the duration of T_I, T_E1 or T_E2 changed with progressive hypoxia among the seven rodent species. There were some similarities in the manner in which T_I, T_E1 and T_E2 changed to produce the shortest T_TOT for each species, however. For the majority of species, when T_TOT was at its shortest, the largest decrease was in T_E2, except for spiny rats. Spiny rats decreased T_I most to produce their shortest T_TOT. Ground squirrels and guinea pigs produced their shortest T_TOT by decreasing T_I and T_E2, while rats decreased in all three phases of the breathing cycle (Figure 6.2).

To determine whether the decreases in V_T, particularly at the highest breathing frequencies and shortest T_TOT, reflected insufficient time for inspiration, we calculated the mean inspiratory airflow rate (represented as V_T/T_I) (Figure 6.3). Again there was much variability across the levels of inspired O_2 in each species. Comparing values for the breaths in each species with the
shortest $T_{TOT}$ we see no overall trend, although when comparing the values at 21% O$_2$ with 9% O$_2$ in each species, we see a decrease in mean inspiratory flow rate in most species except gerbils, hamsters and guinea pigs (Figure 6.3).

When comparing the ratio of the time spent inspiring to the time spent expiring (I to E), there was typically an increase in I/E at low levels of hypoxia, followed by a decrease in I/E as hypoxia became more severe. For certain species (hamsters, ground squirrels, rats and guinea pigs), there was a secondary increase in I/E at the highest levels of hypoxia (9% O$_2$; Figure 6.4).

Overall, the changes to the inspiratory duty cycle ($T_I/T_{TOT}$), the expiratory braking duty cycle ($T_{E1}/T_{TOT}$) and the expiratory pause duty cycle ($T_{E2}/T_{TOT}$) were minor with progressive hypoxia (Figure 6.5).

6.4 Discussion

The overall responses to hypoxia recorded in this study were very modest suggesting that even at 9% inspired O$_2$ these species were still on the relatively flat portion of the exponential hypoxic ventilatory response curve. In the majority of species, for the increases in breathing frequency we did see with progressive hypoxia, AE was never recruited, even at the highest level of respiratory drive (9% inspired O$_2$), suggesting that passive expiration was always sufficient to accommodate these increases in breathing frequency.

Any increase in $f_R$ must be due to an overall decrease in $T_{TOT}$. Some species maintained this decrease in $T_{TOT}$ across all levels of progressive hypoxia (i.e. rats) while some species decreased $T_{TOT}$ to a minimum level only for it to increase again at more severe hypoxic levels (i.e. hamsters). The most substantial decrease in $T_{TOT}$ was typically due to a decrease in $T_{E2}$ as hypothesized. If $T_{TOT}$ must decrease, then maintaining any potential expiratory pause would be
counterintuitive. This substantial decrease in $T_{TOT}$ was also due to a reduction in expiratory braking ($T_{E1}$) in ground squirrel and rats. This decrease in $T_{E1}$ was also hypothesized, as expiratory braking acts to slow expiration. By reducing $T_{E1}$, the antagonistic inspiratory activity that apposes the passive collapse of the respiratory system is removed, resulting in a faster passive expiration. Interestingly, a decrease in $T_{E1}$ was not common among the species studied. Perhaps, the respiratory drive had not increased adequately in the majority of species to require a decrease in $T_{E1}$. The decrease in $T_{TOT}$ was also due to a decrease in $T_1$ for spiny rats, ground squirrels, rats, and guinea pigs. As a result, for theses species, the observed increase in breathing frequency (decrease in $T_{TOT}$) was due to a faster inspiration in addition to a faster expiration. For some species (spiny rats, ground squirrels, and rats), $T_1$ began to increase as the level of hypoxia became more severe (reaching 11 and 9% $O_2$). While $T_{E1}$ and $T_{E2}$ also began to increase with progressive hypoxia, they typically both remained reduced compared to normoxic levels.

The ratio of I/E increased initially followed by a decrease, this decrease was typically followed by an increase as levels of hypoxia became even more severe (11% and 9% $O_2$; Figure 6.4). This suggests that while the initial increase in breathing frequency was due to a more pronounced decrease in $T_E$, as hypoxic exposure progressed there was a greater reduction in $T_1$. Finally, at the highest levels of hypoxia (11% and 9% O2), any maintained increase in breathing frequency (compared to normoxia) was due to a substantial decrease in $T_E$. These results are similar to those of Smith et al., (1989), which showed that when comparing control (air) condition to hypoxic conditions (9% inspired $O_2$) the decrease in $T_1$ is much smaller (1.9s vs. 1.3 s) compared to the decrease in $T_E$ (2.4s vs. 1.1s) for awake dogs.

Interestingly, there were no clear trends in the changes observed to the proportion of $T_1$, $T_{E1}$ and $T_{E2}$ in the overall breathing cycle ($T_{TOT}$) with progressive hypoxia. This is quite different
from the changes in $T_I/T_{TOT}$, $T_{E1}/T_{TOT}$ and $T_{E2}/T_{TOT}$ observed under progressive hypercapnia (Chapter 5). It appears as though the changes to the duration of $T_I$, $T_{E1}$, and $T_{E2}$ with progressive hypoxia are proportional, resulting in a similar ratio of I, E1 and E2 across all hypoxic levels. These results are consistent with those of Smith et al., (1989), which showed that while $T_I$, $T_{E1}$ (measured from the costal diaphragm) and $T_{E2}$ all decrease with hypoxia (9% inspired $O_2$), the calculated duty cycles of $T_I$ (0.44 vs. 0.54), $T_{E1}$ (0.06 vs. 0.06) and $T_{E2}$ (0.50 vs. 0.40) remained fairly consistent compared to control (air) conditions. From a neural control perspective, this suggests that the rhythm generators for the I, E1 and E2 phases have a firing ratio that remains relatively constant. As a result, while the duration of each phase is modified to increase breathing frequency, each phase retains a similar firing ratio.

6.4.1 Conclusion

This comparative analysis of rodents reveals that progressive hypoxia at these levels does not recruit active expiration; passive expiration was always sufficient to accommodate these increases in breathing frequency. This suggests that airway resistance must be low enough and respiratory system compliance adequate to provide sufficient passive expiration to allow for the increases in breathing frequency observed during hypoxic exposure. Future studies will examine whether differences in respiratory mechanics account for the differences in breathing patterns demonstrated by these nine species.
Figure 6.1 Effect of progressive hypoxia on the tidal volume and breathing frequency of seven rodent species. Tidal volume (purple bars) and breathing frequency (green bars) of seven rodent species when exposed to 21%, 19%, 17%, 15%, 13%, 11% and 9% progressive O₂. The black hatched bars represent the O₂ level that produced the shortest total breathing cycle time (T_{TOT}) in each species. Bars represent mean values ± S.E.M.
Figure 6.2 Effect of progressive hypoxia on the duration of the breathing cycle phases of seven rodent species. The inspiratory phase ($T_I$, blue bars), expiratory braking phase ($T_{E1}$, green bars) and secondary expiratory phase ($T_{E2}$, yellow bars) of the diaphragm are presented for seven rodent species exposed to 21%, 19%, 17%, 15%, 13%, 11% and 9% progressive O$_2$. The black hatched bars represent the O$_2$ level that produced the shortest total breathing cycle time ($T_{TOT}$) in each species. Bars represent mean values ± S.E.M.
Figure 6.3 Effect of progressive hypoxia on $V_T/T_1$ of seven rodent species. $V_T/T_1$ is an estimate of airflow for the seven rodent species exposed to 21%, 19%, 17%, 15%, 13%, 11% and 9% progressive O$_2$. The black hatched bars represent the O$_2$ level that produced the shortest total breathing cycle time ($T_{TOT}$) in each species. Bars represent mean values ± S.E.M.
Figure 6.4 Effect of progressive hypoxia on the ratio of inspiration to expiration (I/E) of seven rodent species. I/E is a comparison between the duration of inspiration (I) to the duration of expiration (E) for seven rodent species exposed to 21%, 19%, 17%, 15%, 13%, 11% and 9% progressive O₂. The black hatched bars represent the O₂ level that produced the shortest total breathing cycle time (Tₜₒₜ) in each species. Bars represent mean values ± S.E.M.
Figure 6.5 Effect of progressive hypoxia on the duty cycles of the breathing phases of seven rodents species. The inspiratory phase (I, blue bars), expiratory braking phase (E1, green bars) and secondary expiratory phase (E2, yellow bars) of the diaphragm are presented as a ratio of the total breathing cycle time ($T_{TOT}$) for seven rodent species. Animals were exposed to 21%, 19%, 17%, 15%, 13%, 11% and 9% progressive $O_2$. 
Chapter 7: Pulmonary mechanics and the presence of AE in rodents: a comparative analysis

7.1 Introduction

The breathing response of mammals is typically defined by three phases: inspiration (I), expiratory braking (E1), and a secondary expiratory phase (E2) during which there is passive expiration and potentially an expiratory pause (Richter, 1996; Feldman and McCrimmon, 2003). Inspiration is an active process, dependent on the contraction of the respiratory muscles (namely the diaphragm and the external intercostal muscles) to draw air into the lungs. Expiration, under eupneic conditions, is typically a passive process. When respiratory drive increases, the abdominal muscles can be recruited to produce an active expiration (AE). Previous studies have shown that hypercapnia recruits the abdominal muscles to assist expiration in a number of rodent species (Chapter 4). During exposure to hypercapnia, there are a number of ways in which AE is recruited by these species. The majority of the species (gerbils, mice, spiny rats hamsters, rats, and capybaras) exhibit a late expiratory (late-E) AE, generating a four-phase breathing cycle that consists of I, E1, E2 and AE. Ground squirrels and guinea pigs appear to exhibit a more severe version of this breathing cycle, exhibiting a full expiration (full-E) AE, generating a two-phase breathing cycle of I followed by AE. Agoutis are unique to the other rodents mentioned, as they exhibit an early expiration (early-E) AE, generating a three-phase breathing cycle that consists of an I, AE, and E2. While the placement of AE varied among these species, there was a consistent increase in tidal volume ($V_T$) along with the appearance of AE within the breathing cycle. Exposure to hypoxia never produced AE (Chapter 5).
The observed differences in when the abdominal muscles are recruited may be a function of the mechanics of the respiratory system. A late-E AE, concurrent with the large increase in $V_T$ will recruit the expiratory reserve volume (ERV). The magnitude of this, as well as the level of drive at which it occurs, may reflect the size of each species ERV. An early-E AE, on the other hand, may be necessary to aid in releasing air during expiration in a system with elevated resistance and/or compliance. The lack of AE during hypoxic exposure may be due to high elastic recoil of the respiratory system, such that no forced expiration is required to speed up the expiratory time ($T_E$) past the passive expiratory time constant ($\tau_E$).

The purpose of this study was to determine if there are any differences in the respiratory mechanics of these rodent species that would explain the observed differences in when and how AE was recruited within the breathing cycle. We hypothesized that 1) all rodent species would have an expiratory reserve volume (ERV) but that species exhibiting the largest increases in tidal volume, or the species that recruited AE at lower levels of respiratory drive would have the largest ERV, 2) that agoutis, which recruit an early-E AE would have a higher resistance and/or a higher compliance than the other rodents that exhibit AE later in the breathing cycle and 3) that $\tau_E$ would be smaller than the $T_E$ observed at elevated breathing frequencies seen in hypoxia in all species, explaining why AE was only seen in association with elevations in tidal volume.

7.2 Methods

All experiments were performed with approval from the University of British Columbia Animal Care Committee (A13-0025), under the guidelines of the Canadian Council for Animal Care (CCAC).
7.2.1 Experimental animals

Adults (n=2) of various species of rodents (gerbils, mice, spiny rats, hamsters, rats (n=6), ground squirrels, guinea pigs, agouti and capybaras) were used in this study. See Table 7.1 for body weights for all species.

7.2.2 Experimental procedure

Rodents were anesthetized with isoflurane or ketamine until the toe pinch and corneal reflexes were abolished. Animals were then tracheostomized through a midline incision and a tracheal cannula was inserted. The animals were placed supine, and pressure was measured from a sidearm of the tracheal cannula with a pressure transducer.

7.2.3 Measuring lung volumes

Stepwise inflation and deflation curves were conducted for all species. TLC was measured by hyper-inflating the lungs to a pressure of 30 cm H$_2$O from FRC. The lungs were deflated from FRC to a pressure of -20 cm H$_2$O to determine ERV.

7.2.4 Measuring static compliance

The following techniques mimic those of Milsom and Vitalis (1984) and Milsom and Reid (1995). The lungs were mildly inflated and allowed to deflate two or three times to establish resting lung volume (functional residual capacity, FRC). The volume of the lungs at FRC then was determined by removing air from the lungs with a syringe connected to the tracheal cannula. The system was then inflated to a pressure of 30 cmH$_2$O and reduced to at least -20 cmH$_2$O. The steady state pressure changes associated with 3 stepwise inflation and deflation
sequences between these pressures were recorded. The relationship between the change in volume and the change in pressure represented the quasi-static compliance curve of the entire respiratory system (RS). After the dynamic compliance measurements (described below), a mid-ventral incision was made and the lungs were exposed. The steady state pressure changes associated with stepwise inflation and deflation sequences of the exposed lungs were then recorded. The relationship between the change in volume with the change in pressure of the exposed lungs represented the quasi-static compliance curve for the lungs (L). The lungs were swabbed with saline during this procedure to prevent drying. The compliance curve of the chest wall (CW) was calculated as the difference between CRS and CL. The compliance of the respiratory system (CRS), lungs (CL) and chest wall (CW) were calculated as the slope of the steepest part of each curve (Figure 7.1).

### 7.2.5 Measuring dynamic compliance

Animals were connected to a respirator and ventilated at a minimum of three different tidal volumes each at five different frequencies (as described in Milsom and Reid (1995)). Dynamic compliance was not recorded for the capybaras, as we did not have access to a pump that was adequate for the lung volumes of this animal. The pressure change during tracheal airflow was measured with a differential pressure transducer connected to a pneumotachograph. All measurements of pressure, airflow and volume were recorded simultaneously. The dynamic volume-pressure curves were generated for every combination of tidal volume and breathing frequency. Dynamic compliance was calculated for each combination of breathing frequency and tidal volume as the slope of the line connecting the points of zero flow on the pressure-volume loops (the points of maximum and minimum volume) (Figure 7.2A). The resistance was
estimated for each combination of breathing frequency and tidal volume by calculating
difference in pressure between two points of isovolume and dividing by the flow (Figure 7.2B).

The passive expiratory time constant ($\tau_E$) was determined for each combination of
breathing frequency and tidal volume by dividing the volume by the peak expiratory flow (as
described by Brunner et al., 1995; Figure 7.3). Breathing frequency and tidal volume
combinations that closely matched breathing frequencies and tidal volume combinations
observed during hypercapnic (Chapter 4) and hypoxic (Chapter 5) exposures in vivo allowed for
assumptive comparisons between $\tau_E$ (calculated from flow-volume curves) and $T_E$ (measured in vivo).

7.3 Results

7.3.1 Static pressure-volume measurements

The body weights of the nine species studied ranged from 50 g to 31 kg (Table 7.1).
Vital capacities ranged from 1.3 to 5% of body volume (assuming a specific gravity of 1.0) and
the expiratory reserve capacity (ERV) ranged from 10.25 ± 1.25 % to 28.00 ± 2.00 % of the
animal’s vital capacity (mean = 21.79 ± 2.10) (Table 7.1, Figure 7.4). The smallest ERVs were in
the guinea pig and rat (10.25 ± 1.25% and 11.12 ± 3.42%, respectively) and the largest in the
ground squirrel, gerbil, and agouti (26.00 ± 1.00%, 27.50 ± 0.50%, and 28.00 ± 2.00%,
respectively). Vital capacity increased with body mass, but the relationship was non-linear
($R^2=0.999$; Figure 7.5).

Figure 7.6 depicts the quasi-static pressure-volume curves for each rodent species. The
compliance of the components of the respiratory system (total respiratory system $C_{RS}$, lung $C_L$
and chest wall $C_{CW}$) varied between species (Table 7.2). When normalized to vital capacity, the
agoutis had the greatest total respiratory system, chest wall and lung compliance of all the species studied.

### 7.3.2 Dynamic pressure-volume measurements

As dynamic respiratory mechanics vary as a function of the tidal volume and breathing frequency, we chose to compare the dynamic compliance among the eight species at three different tidal volume and breathing frequency combinations (dynamic compliance of the capybaras could not be measured as we did not have a ventilator capable of generating large enough volumes).

There was no substantial change in the dynamic compliance of the passive respiratory (total) system of these rodent species as $V_T$ increased; the only exceptions being a mild decrease in compliance in gerbils and rats, and a mild increase in compliance for spiny rats (Figure 7.7). There was no effect of breathing frequency on compliance of the passive respiratory system, except for hamsters at the highest tidal volume (Figure 7.7).

There was also no substantial change in the resistance to flow in the passive respiratory (total) system for these rodents species as $V_T$ increased, with the exception of mice and ground squirrels, which had a slight increase in resistance, and agoutis, which had a slight decrease in resistance (Figure 7.8). There was no substantial effect of increasing breathing frequency on the resistance to flow of the passive respiratory system (Figure 7.8).

We compared the compliance and resistance to flow of the total passive system measured at the maximal $V_T$ paired to pump frequencies that closely matched the breathing frequencies that initially produced AE under hypercapnic conditions for each species (data from Chapter 4; Table 7.3). For the three smallest species, the dynamic compliance at these combinations was
roughly the same as the static compliance (see Table 7.2). For the other five species, the system stiffened when ventilated with the dynamic compliance becoming 2 to 5 times less than the static compliance at these combinations of $V_T$ and $f_R$. The agoutis had the largest compliance and lowest resistance of all species. When normalized for VC, however, this species difference in dynamic compliance was eliminated indicating that the greater compliance was due to the greater vital capacity.

The passive expiratory time constant was determined for each animal at a specific breathing frequency and tidal volume that closely matched the observed breathing frequencies and tidal volumes exhibited by theses species under *in vivo* hypoxic conditions (data from Chapter 5). The calculated $\tau_E$ for each animal ranged from 0.09 ± 0.030 seconds to 0.24 ± 0.005 seconds. When compared with data for *in vivo* $f_R$ and $V_T$, $\tau_E$ was shorter than $T_E$ for every animal (Figure 7.6). On average, $\tau_E$ was 57.25 ± 8.25% shorter than $T_E$. The major exception to this was the agoutis, which had the highest $\tau_E$ value, representing 94.55% of $T_E$ at their highest breathing frequency. Interestingly, $T_{E1}$ was shorter than $\tau_E$ for most species (Figure 7.9), particularly for the agoutis. This indicates that expiratory airflow must continue into $T_{E2}$ in these species. The only exceptions were the mice and rats. In these species, presumably the expiratory braking sustains expiratory airflow until the end of $T_{E1}$.

### 7.4 Discussion

This study was designed to determine whether differences and/or similarities in the pulmonary mechanics of a number of rodent species could explain differences in the recruitment of active expiration (AE).
We had hypothesized that AE occurring in late-E would serve to enhance VT by drawing on the ERV. If the expiratory airflow has ended and the lungs are stable at FRC, then a late-E AE would serve to expel air from the ERV enhancing the size of the next breath. When we compare our measures of $\tau_E$ and $T_E$ for each species, this would appear to be the case; in all instances $T_E$ was longer than $\tau_E$ even at the highest breathing frequencies recorded under hypoxic conditions. This confirms that the elastic recoil of the system is sufficient to passively exhale air from the lungs without an active expiration even under hypoxic conditions. The lungs should have been at FRC at the end of the expiratory phase. The one exception was the agouti where $\tau_E$ closely approaches $T_E$ at the highest breathing frequencies.

What was surprising was that most of the species had a $\tau_E$ that was larger than $T_{E1}$. This suggests that the expiratory braking ($T_{E1}$) activity does not occur throughout the entirety of expiratory airflow. In our study, the measurement of expiratory braking is only based on the post-inspiratory activity of the diaphragm. As a result, the total expiratory braking activity of the animal (which consists of both diaphragm and laryngeal muscle activity) may be longer than our measured values. It has been shown that the relevant importance of the diaphragm and laryngeal muscles appears to change with respiratory stimuli. Under eupneic conditions, the expiratory braking phase of adult cats is more dependent on post-inspiratory diaphragm activity than laryngeal (posterior cricoarytenoid muscle, PCA) muscle activity (Bartlett et al., 1973). However, with progressive hypercapnia, there is a decrease in post-inspiratory diaphragm activity and a substantial increase in the PCA expiratory braking activity (Bartlett et al., 1973). From representative traces within their paper, it appears as though expiratory braking does not continue throughout the entirety of the expiratory airflow, however, as the post-inspiratory EMG activity terminated before the complete emptying of the lungs (Bartlett et al., 1973). This
suggests that our conclusion that expiratory braking does not occur throughout the entirety of expiratory airflow is valid.

The difference in the size of the ERV among species reflects the balance between lung and chest wall mechanics that establish FRC. In our study group, the ERV ranged from 10% to almost 30% of vital capacity. There was no correlation, however, between the size of the ERV and the level of CO₂ at which AE was recruited to enhance tidal volume. Thus even though there was a correlation between the level of CO₂ at which tidal volume began to increase significantly and the appearance of AE (Chapter 5), this was independent of the size of the ERV to draw upon. We would hypothesize that the smaller the ERV, the less likely it would be that an animal would use AE to enhance tidal volume and the larger the ERV, the more likely it would be to do so. Clearly, this was not the case over the range of ERVs in the species in the present study. The data would indicate that the ERV was sufficiently large in all cases.

As for where AE appears in the breathing cycle, the results of this study suggest this may reflect pulmonary mechanics. Within the literature, there is evidence that AE is recruited during early-E to overcome flow resistance. Campbell found that human subjects consistently recruited the abdominal muscles during expiration when presented with an increase in expiratory resistance (from 0 cmH₂O to 17.5 cmH₂O; 1957). For the agoutis where AE occurred in early-E, the dynamic compliance was greater and flow resistance lower than in all the other species. However, while a high compliance would reduce elastic recoil forces driving expiration, the low resistance would allow higher rates of expiratory flow and these two factors appear to be off-setting as the \( \tau_E \) for the agoutis was similar to that for all other species. As mentioned above, however, \( \tau_E \) closely approaches \( T_E \) at higher breathing frequencies in the agouti and \( T_{E1} \) is negligible. This indicates that AE is coincident with expiratory airflow and is required to produce
full expiration within the time available. This seems the most plausible explanation for the early-E AE in this species.

The short-tailed opossum also exhibits an early-E AE, even under eupneic conditions (Reilly and White, 2009). It would be useful to determine whether this species has either a large expiratory air flow resistance or a $\tau_E$ that approaches the eupneic $T_E$. It was proposed that the abdominal activity exhibited by the short-tailed opossum helps to maintain the high ventilation rates (Reilly and White, 2009).

The typical late-E AE is generated by late-E neural activity within the parafacial respiratory group/retro-trapezoidal nucleus (RTN/pFRG) in the rostral medulla. This activity is inhibited under eupneic conditions but the inhibition is removed by hypercapnic chemosensory drive; i.e. expression of AE requires disinhibition of these neurons (Pagliardini et al., 2011). We have previously shown that the Kölliker-Fuse nucleus (KF) in the pons acts as a timekeeper for the onset of the late-expiratory (late-E) AbN activity observed during the hypercapnic response (Chapter 3). Presumably the differences in the level of CO$_2$ at which this occurs in the present study reflect either differences in CO$_2$ chemosensitivity, or differences in the inhibitory threshold that has to be overcome for AE to be expressed.

The basis of the appearance of AE in early-E in the agouti versus late-E in the other species is not clear. Early-E AE, also refereed to as post-I abdominal activity, has been observed in situ, with the working heart brainstem preparation (Abdala et al., 2009). It was suggested that this post-I abdominal activity acts to provide muscle tone that assists with the release of air during expiration (Abdala et al., 2009), which is consistent with what we have proposed for the agouti. Models of the neural control of breathing have highlighted the role of the Bötzinger complex post-I neurons in stimulating rostral and caudal VRG neurons to generate early-E
abdominal muscle activity (Smith et al., 2011) and this may arise due to excitatory drive from the RTN/pFRG and the pons (Abdala et al., 2009). The data in Chapter 3 lead to the conclusion that the KF plays a key role in inhibiting the emergence of expiratory abdominal activity, acting as a timekeeper for active expiration, but the mechanisms underlying how the appearance and duration of AE are controlled remain unknown.

We hypothesized that 1) all rodent species would have an expiratory reserve volume (ERV) but that species exhibiting the largest increases in tidal volume and recruiting AE at lower levels of respiratory drive would have the largest ERV. This was not the case. While all species had a significant ERV, three was no correlation between the size of the ERV and the level of CO₂ at which it was recruited. 2) We hypothesized that agoutis, which recruit an early-E AE, would have a higher resistance and/or a higher compliance than the other rodents that exhibit AE later in the breathing cycle. They did have greater static and dynamic compliances, attributable to having a larger vital capacity, but had a lower resistance to expiratory air flow. 3) We hypothesized that τₑ would be smaller than the Tₑ observed at elevated breathing frequencies seen in hypoxia in all species, explaining why AE was only seen in association with elevations in tidal volume and this was the case for all species except the agouti. 4) τₑ closely approached Tₑ at higher breathing frequencies in the agouti and Tₑ⊥ was negligible suggesting that AE was coincident with expiratory airflow and was required to produce full expiration within the time available in this species.
Figure 7.1 Schematic representation of typical mammalian static pressure-volume deflation curves. The curve of the total respiratory system (black line) is the sum of the curve of the chest wall alone (blue dashed line) and the lungs alone (pink dashed line). The static compliance of each curve was calculated from the steepest part of each curve (yellow bar). The horizontal dashed line represents the functional residual capacity (FRC). FRC is volume at which the total respiratory system passes by zero pressure.
Figure 7.2 Schematic diagrams illustrating how measurements of dynamic compliance and resistance were obtained from the pressure-volume curves. A) The slope of the dashed line represents that dynamic compliance of the system. B) The pressure difference between the two grey dots was used to calculate the resistance of the system.
Figure 7.3 Schematic diagram illustrating how the passive expiratory time constant was obtained from a flow-volume loop. The grey dot represents the peak expiratory flow. Dividing the volume by the peak expiratory flow provided an estimate of the passive expiratory time constant.
Figure 7.4 Average expiratory reserve volume as a percentage of the vital capacity of nine rodent species. The expiratory reserve volume is the amount of expiratory space in the lungs following passive expiration and it is represented here relative to vital capacity. Bars represent mean values ± S.E.M.
Figure 7.5 Relationship between body mass (kg) and vital capacity (mL). Non-linear regressions are presented for data points of nine rodent species. The box highlights the non-linear relationship of the seven smallest rodent species. Data points represent mean values ± S.E.M.
Figure 7.6 Average static compliance curves of nine rodent species. The curve of the respiratory system (black line) is the sum of the curve of the chest wall alone (blue dashed line) and the lungs alone (pink dashed line). The horizontal dotted line identifies the functional residual volume (FRC) for each species.
Figure 7.7 Effect of increasing tidal volume on dynamic compliance for eight rodent species. Values for three different breathing frequencies (represented by different colored data points, actual frequencies are identified on each graph) at three tidal volumes for each rodent species. Data points represent mean values ± S.E.M.
Figure 7.8 Effect of increasing tidal volume on resistance for eight rodent species. Values for three different breathing frequencies (represented by different colored data points, actual frequencies are identified on each graph) at three tidal volumes for each rodent species. Data points represent mean values ± S.E.M.
Figure 7.9 Comparison of the passive expiratory time constant ($\tau_E$), expiratory braking phase duration ($T_{E1}$) and total expiratory phase duration ($T_E$) for eight rodent species. Bars represent mean values ± S.E.M.
Table 7.1 Volumes of the components of the respiratory system for nine rodent species. Values are presented in absolute terms (mL) and as a percentage of the animal’s vital capacity (%). Values represent mean ± S.E.M.

<table>
<thead>
<tr>
<th>Species</th>
<th>N</th>
<th>Mass</th>
<th>$V_T$</th>
<th>IRV</th>
<th>ERV</th>
<th>VC</th>
<th>$V_T$</th>
<th>IRV</th>
<th>ERV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>kg</td>
<td>mL</td>
<td>mL</td>
<td>mL</td>
<td>mL</td>
<td>mL</td>
<td>% VC</td>
<td>% VC</td>
<td>% VC</td>
</tr>
<tr>
<td>Gerbil</td>
<td>2</td>
<td>0.05 ± 0.004</td>
<td>0.33 ± 0.03</td>
<td>0.73 ± 0.01</td>
<td>0.28 ± 0.01</td>
<td>1.00 ± 0.00</td>
<td>33.25 ± 3.07</td>
<td>72.50 ± 0.50</td>
<td>27.50 ± 0.50</td>
</tr>
<tr>
<td>Mouse</td>
<td>2</td>
<td>0.06 ± 0.001</td>
<td>0.40 ± 0.05</td>
<td>0.62 ± 0.17</td>
<td>0.18 ± 0.07</td>
<td>0.80 ± 0.10</td>
<td>51.97 ± 13.01</td>
<td>76.25 ± 11.25</td>
<td>23.75 ± 11.25</td>
</tr>
<tr>
<td>Hamster</td>
<td>2</td>
<td>0.12 ± 0.00</td>
<td>0.83 ± 0.01</td>
<td>4.86 ± 0.21</td>
<td>1.14 ± 0.21</td>
<td>6.00 ± 0.00</td>
<td>13.76 ± 0.18</td>
<td>81.00 ± 3.50</td>
<td>19.00 ± 3.50</td>
</tr>
<tr>
<td>Spiny Rat</td>
<td>2</td>
<td>0.13 ± 0.002</td>
<td>1.07 ± 0.00</td>
<td>2.03 ± 0.65</td>
<td>0.57 ± 0.05</td>
<td>2.60 ± 0.60</td>
<td>43.44 ± 10.02</td>
<td>76.50 ± 7.50</td>
<td>23.50 ± 7.50</td>
</tr>
<tr>
<td>Ground Squirrel</td>
<td>2</td>
<td>0.32 ± 0.03</td>
<td>2.32 ± 0.23</td>
<td>7.78 ± 0.48</td>
<td>2.73 ± 0.02</td>
<td>10.50 ± 0.50</td>
<td>22.21 ± 3.24</td>
<td>74.00 ± 1.00</td>
<td>26.00 ± 1.00</td>
</tr>
<tr>
<td>Rat</td>
<td>6</td>
<td>0.37 ± 0.01</td>
<td>2.76 ± 0.09</td>
<td>9.49 ± 0.60</td>
<td>1.18 ± 0.60</td>
<td>10.67 ± 0.49</td>
<td>29.05 ± 2.82</td>
<td>88.88 ± 5.92</td>
<td>11.12 ± 3.42</td>
</tr>
<tr>
<td>Guinea Pig</td>
<td>2</td>
<td>0.46 ± 014</td>
<td>3.41 ± 1.05</td>
<td>10.35 ± 2.39</td>
<td>1.15 ± 0.11</td>
<td>11.50 ± 2.50</td>
<td>26.11 ± 1.46</td>
<td>89.75 ± 1.25</td>
<td>10.25 ± 1.25</td>
</tr>
<tr>
<td>Agouti</td>
<td>2</td>
<td>3.10 ± 0.10</td>
<td>4.68 ± 0.46</td>
<td>68.90 ± 19.90</td>
<td>26.10 ± 5.10</td>
<td>95.00 ± 25.00</td>
<td>5.36 ± 0.36</td>
<td>72.00 ± 2.00</td>
<td>28.00 ± 2.00</td>
</tr>
<tr>
<td>Capybara</td>
<td>2</td>
<td>31.40 ± 0.60</td>
<td>18.96 ± 6.0</td>
<td>449.50 ± 116.13</td>
<td>125.50± 93.51</td>
<td>575.00 ± 50.00</td>
<td>3.47 ± 0.05</td>
<td>77.00 ± 19.09</td>
<td>23.00 ± 19.09</td>
</tr>
</tbody>
</table>

* $V_T$ calculated using equation from Stahl, 1967 based on mass.
Table 7.2 Static compliance for nine rodent species. Values presented for the respiratory system (RS), chest wall (CW) and the lungs (L). The compliance is presented for each species in absolute terms (ml cmH₂O⁻¹) and as a percent of the animal’s vital capacity (%VC cmH₂O⁻¹).

<table>
<thead>
<tr>
<th>Species</th>
<th>C&lt;sub&gt;RS&lt;/sub&gt;</th>
<th>C&lt;sub&gt;L&lt;/sub&gt;</th>
<th>C&lt;sub&gt;CW&lt;/sub&gt;</th>
<th>C&lt;sub&gt;RS&lt;/sub&gt;</th>
<th>C&lt;sub&gt;L&lt;/sub&gt;</th>
<th>C&lt;sub&gt;CW&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mL cm H₂O⁻¹</td>
<td>mL cm H₂O⁻¹</td>
<td>mL cm H₂O⁻¹</td>
<td>% VC cm H₂O⁻¹</td>
<td>% VC cm H₂O⁻¹</td>
<td>% VC cm H₂O⁻¹</td>
</tr>
<tr>
<td>Gerbil</td>
<td>0.04 ± 0.03</td>
<td>0.07 ± 0.01</td>
<td>0.08 ± 0.06</td>
<td>3.85 ± 2.78</td>
<td>6.73 ± 0.88</td>
<td>7.67 ± 6.36</td>
</tr>
<tr>
<td>Mouse</td>
<td>0.04 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td>0.11 ± 0.06</td>
<td>4.80 ± 0.16</td>
<td>6.09 ± 0.38</td>
<td>15.16 ± 9.51</td>
</tr>
<tr>
<td>Hamster</td>
<td>0.40 ± 0.03</td>
<td>0.56 ± 0.09</td>
<td>1.45 ± 0.19</td>
<td>6.60 ± 0.55</td>
<td>8.63 ± 2.20</td>
<td>11.08 ± 9.98</td>
</tr>
<tr>
<td>Spiny Rat</td>
<td>0.16 ± 0.05</td>
<td>0.16 ± 0.08</td>
<td>1.74 ± 0.12</td>
<td>6.07 ± 0.46</td>
<td>5.87 ± 1.57</td>
<td>5.55 ± 3.40</td>
</tr>
<tr>
<td>Ground Squirrel</td>
<td>0.48 ± 0.02</td>
<td>0.57 ± 0.27</td>
<td>0.83 ± 0.01</td>
<td>4.84 ± 0.67</td>
<td>5.46 ± 2.11</td>
<td>8.40 ± 0.72</td>
</tr>
<tr>
<td>Rat</td>
<td>0.68 ± 0.06</td>
<td>0.98 ± 0.20</td>
<td>1.22 ± 0.28</td>
<td>5.29 ± 1.18</td>
<td>7.43 ± 2.06</td>
<td>12.21 ± 3.62</td>
</tr>
<tr>
<td>Guinea Pig</td>
<td>0.44 ± 0.28</td>
<td>0.59 ± 0.10</td>
<td>0.32 ± 0.10</td>
<td>4.54 ± 3.44</td>
<td>5.60 ± 2.09</td>
<td>3.14 ± 1.53</td>
</tr>
<tr>
<td>Agouti</td>
<td>7.08 ± 0.81</td>
<td>13.87 ± 1.62</td>
<td>19.34 ± 9.58</td>
<td>9.06 ± 3.23</td>
<td>15.20 ± 2.30</td>
<td>24.72 ± 16.59</td>
</tr>
<tr>
<td>Capybara</td>
<td>34.40 ± 8.31</td>
<td>16.75 ± 14.02</td>
<td>21.38 ± 13.92</td>
<td>6.15 ± 1.98</td>
<td>3.81 ± 3.03</td>
<td>3.96 ± 2.77</td>
</tr>
</tbody>
</table>
Table 7.3 Dynamic compliance and resistance for eight rodent species. Values are presented for dynamic compliance (represented as absolute and as a percentage of the vital capacity), resistance and passive expiratory time constant ($\tau_E$, sec). The dynamic compliance and resistance were determined for the combination of breathing frequency at which AE first appeared during progressive hypercapnia in vivo (Chapter 5) and the largest tidal volume in our pump ventilation series. Values represent means ± S.E.M.

<table>
<thead>
<tr>
<th>Species</th>
<th>CO$_2$ % that recruits AE</th>
<th>AE placement</th>
<th>$C_{RS}$</th>
<th>$R_{RS}$</th>
<th>$C_{RS}$</th>
<th>$\tau_E$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gerbil</td>
<td>8%</td>
<td>Late-E</td>
<td>0.057 ± 0.002</td>
<td>5.72 ± 2.46</td>
<td>5.74 ± 0.24</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>Mouse</td>
<td>8%</td>
<td>Late-E</td>
<td>0.08 ± 0.03</td>
<td>4.43 ± 0.40</td>
<td>10.27 ± 2.73</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>Hamster</td>
<td>6%</td>
<td>Late-E</td>
<td>0.53 ± 0.16</td>
<td>1.49 ± 0.67</td>
<td>8.84 ± 2.61</td>
<td>0.18 ± 0.02</td>
</tr>
<tr>
<td>Spiny Rat</td>
<td>10%</td>
<td>Late-E</td>
<td>0.056 ± 0.004</td>
<td>2.56 ± 0.069</td>
<td>2.27 ± 0.58</td>
<td>0.25 ± 0.005</td>
</tr>
<tr>
<td>Ground Squirrel</td>
<td>6%</td>
<td>Full-E</td>
<td>0.41 ± 0.13</td>
<td>1.80 ± 0.07</td>
<td>3.87 ± 1.04</td>
<td>0.16 ± 0.03</td>
</tr>
<tr>
<td>Rat</td>
<td>8%</td>
<td>Late-E</td>
<td>0.34 ± 0.05</td>
<td>0.69 ± 0.15</td>
<td>3.34 ± 0.60</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td>Guinea Pig</td>
<td>6%</td>
<td>Full-E</td>
<td>0.15 ± 0.05</td>
<td>0.71 ± 0.27</td>
<td>1.44 ± 0.72</td>
<td>0.16 ± 0.03</td>
</tr>
<tr>
<td>Agouti</td>
<td>10%</td>
<td>Early-E</td>
<td>1.29</td>
<td>0.01</td>
<td>1.08</td>
<td>0.18</td>
</tr>
</tbody>
</table>
Chapter 8: Conclusion

8.1 Respiratory stimuli modify the breathing cycle differently

Hypoxia and hypercapnia certainly modify the breathing pattern differently (Rebuck et al., 1976; Cragg and Drysdale, 1983). The typical hypercapnic ventilatory response is characterized by a substantial increase in tidal volume ($V_T$) with a less pronounced increase in breathing frequency ($f_R$). Alternatively, the hypoxic ventilatory response is characterized by a substantial increase in $f_R$, with little change to $V_T$ (Cragg and Drysdale, 1983). The underlying neural responses to hypercapnia and hypoxia are clearly different (see Baker et al., 2001 for review), with hypercapnia influencing the amount of spinal nerve motor output, and hypoxia influencing the frequencies of the respiratory rhythm generators. This suggests that stimulation of the peripheral ($O_2$, $CO_2$, pH) and central ($CO_2$, pH) chemoreceptors are integrated and processed differently, resulting in different respiratory muscle recruitment.

8.2 The neural sites that generate breathing

Any observed changes in tidal volume and breathing frequency are due to changes in the intensity and/or duration of respiratory muscle contractions (Braun, 1990). All respiratory muscle contractions that power the mammalian aspiration pump are under the control of highly organized neurons within the medulla. A region within the medulla, known as the ventral respiratory column (VRC), is critical for the generation of eupneic breathing. Within the VRC lies the preBötzinger Complex (preBötC) and the Bötzinger Complex (BötC). The preBötC is thought to be the inspiratory (I) oscillator, critical and sufficient for the generation of inspiratory rhythmic activity (Smith et al., 1991). The BötC contains the eupneic expiratory (E) neurons.
Within the BötC there are neurons that fire during the first portion of expiration (E1 or post-inspiratory (post-I) neurons), and neurons that fire during the end of expiration (E2 or augmenting expiratory (aug-E) neurons; Smith et al., 2009). These I, E1 and E2 neurons project to, and synapse on, the cranial and spinal motor nerves to power the appropriate respiratory muscles at the appropriate times (Smith et al., 2009).

These neural regions are critical for generating the three-phase eupneic breathing cycle: inspiration (I), expiratory braking (E1) and the second expiratory phase (E2; Richter, 1982). Inspiration is always active, dependent on the contraction of the inspiratory muscles (the diaphragm and at times the external intercostal muscles) to draw air into the lungs. The first expiratory phase (E1), also known as expiratory braking, is characterized by the prolonged contraction of the inspiratory muscles (diaphragm and the external intercostal muscles; Chapter 2) into the expiratory phase, in addition to contraction of the laryngeal muscles (Gautier et al., 1973). This phase acts to slow the rate at which air leaves the lungs, and is thought to prolong gas exchange (Richter and Smith, 2014). The second phase of expiration (E2) is characterized by passive expiratory airflow and an expiratory pause. The duration of the passive expiratory airflow and expiratory pause varied with species and with respiratory conditions (Chapter 2).

When respiratory drive is elevated, expiration can transition from passive to active. During an active expiration (AE), the abdominal and internal intercostal muscles are recruited to force air out of the lungs (see Feldman et al., 2013 for review). This activity appears to be dependent on the neuronal activity of a separate region of the medulla, known as the retrotrapezoid nucleus/parafacial respiratory group (RTN/pFRG). The current hypothesis is that the pFRG is the expiratory rhythm generator, critical for the production of expiratory abdominal
muscle activity when respiratory drive is elevated (Onimaru et al., 1987; 1988, Onimaru and Homma, 2003; Janczewski and Feldman, 2006).

8.3 The critical role of the pons and afferent vagal feedback in modifying the breathing response

Both the pons and afferent vagal feedback play a role in coordinating the transition between the inspiratory and expiratory phases (Lumsden, 1923; Stella, 1983). The pons is critical for the termination of the inspiratory activity and the commencement of the post-inspiratory activity (Mörschel and Dutschmann, 2009). The Kölliker-Fuse (KF) and medial parabrachial nucleus are thought to be critical to providing an inspiratory off-switch, terminating I and commencing E1 activity (Mörschel and Dutschmann, 2009). Inhibiting the KF with isoguvacine eliminates post-inspiratory activity (Dutschmann and Herbert, 2006; Bautista and Dutschmann 2014; Chapter 3). Similarly, complete transection of the pons eliminates the post-inspiratory phase of the breathing cycle, transforming the eupneic three-phase cycle (I, E1 and E2) into a two-phase breathing cycle (I, E) with no post-inspiratory (E1) activity (Abdala et al., 2009).

Afferent vagal feedback from stretch receptors in the lungs was discovered to play a role in the Hering-Breuer reflex, which ensures the lungs do not hyper-inflate or hyper-deflate (Breuer, 1868; Hering, 1868; see Moore, 1926). As afferent vagal feedback is critical for controlling the duration of the inspiration and expiration phases, bilateral vagotomy typically prolongs inspiration and produces a slow and deep breathing pattern (Amphibians: Reid et al., 2000. Reptiles: Milsom, 1984; Milsom Jones, 1980. Mammals: Fedorko et al., 1988; Harris Milsom, 2001).
8.4 The importance of disinhibition in the recruitment of the respiratory muscles of the rat

Not surprisingly, the diaphragm was always active in our experiments (Chapter 2, 5, and 6). As a result, the DIA$_m$ activity consisted of an inspiratory phase (I), an expiratory braking phase (E1), where diaphragm activity would continue into the early expiratory phase, followed by a secondary phase where there was no diaphragm activity during expiration (E2). No intercostal muscle activity was recorded under eupneic conditions for unanesthetized rats with vagi intact (Chapter 2). It is important to clarify that we only recorded intercostal muscle activity from the lower rib cage (thoracic space (T) 10-T13). As levels of hypercapnia became more severe, however, the activity of the INT$_m$ became more prominent during inspiration. This did not occur for hypoxic (unanesthetized) conditions, suggesting that CO$_2$ might play a role in generating this motor output.

Interestingly, when rats were anesthetized with urethane, the activity of the INT$_m$ was present during inspiration under eupneic conditions and at all levels of progressive hypercapnia and hypoxia. Based on the evidence that urethane depresses excitatory and not inhibitory pathways (Hara et al., 2002; Daló and Hackman, 2013), we speculate that under eupneic conditions, INT$_m$ activity was inhibited and that urethane anesthetic removed this inhibition. The evidence that CO$_2$ and anesthetics contributed to this disinhibition of INT$_m$ activity, suggests that there may be multiple mechanisms in place for the recruitment of the (lower) intercostal muscles. While the source(s) of the INT$_m$ activity inhibition remain unknown, it appeared to influence external intercostal (inspiratory) muscle activity more than the internal intercostal (expiratory) muscle activity.
It is also important to note that when \( \text{INT}_m \) activity was present its activity often continued past the inspiratory phase into the expiratory braking phase. To our knowledge, this is the first documented case of the caudal external intercostal muscles, along with the rostral intercostal muscles (Berdah and De Troyer, 2001), contributing to expiratory braking, in addition to a number of laryngeal muscles (Bartlett et al, 1973; England et al, 1985; O’Halloran et al., 1999), and the diaphragm (D’Angelo et al., 2010; Subramanian and Holstege, 2011; Chapter 2; Chapter 5, Chapter 6).

Phasic abdominal muscles, which produce active expiration, were also inhibited under eupneic conditions (Chapter 2, Chapter 5). Pagliardini et al., (2011) found that when the RTN/pFRG was disinhibited chemically with a GABA\(_A\) antagonist, there was a substantial increase in abdominal muscle activity at the end of expiration. We found a similar disinhibition with hypercapnia \textit{in vivo}, as no phasic expiratory abdominal activity was present under eupneic conditions, but as levels of hypercapnia became more severe (8% and 10% CO\(_2\)), expiratory abdominal AE was recorded. Hypercapnia consistently recruited AE in a number of species (Abe et al., 1996; rats: Sherrey et al., 1988; Janczewski et al., 2002; Abdala et al., 2009; Taccola et al., 2007; Chapter 2; a number of rodent species: Chapter 5; dogs: De Troyer et al., 1989; goats: O’Halloran et al., 1999), highlighting the role of CO\(_2\) chemoreception in providing a source of disinhibition. Interestingly, stimulation of central CO\(_2\) chemoreceptors in isolation did not seem sufficient to generate AE, as AE was not observed with the \textit{in vitro} preparation (Chapter 3). The reason for this remains unknown. It could be that the source of disinhibition of the RTN/pFRG was not present within the \textit{in vitro} brainstem-spinal cord preparation, or it could be due to the age of the \textit{in vitro} preparation (the neonatal hypercapnic response is reduced; Fong \textit{et al.}, 2008; Studden et al., 2001; Serra et al., 2001; Wickstrom et al., 2002). Alternatively, it could be that
the RTN/pFRG was damaged by to the hyperoxic artificial cerebral spinal fluid due to the more superficial position of the RTN/pFRG (~250 µm; Chen et al., 2013) compared to the preBötC (~400-500 µm; Fong et al., 2008; Chen et al., 2013; O2 gradient provided by Fong et al., 2008). In contrast to our results, Iizuka was able to record expiratory abdominal activity from the T13 and L5 when using the *in vitro* brainstem spinal cord preparation (2003). Unfortunately, the reason for the discrepancy in the ability to record active expiratory activity with this preparation remains unclear. Given the great variability of stimuli that can generate AE (hypercapnia: Abdala *et al.*, 2009; Chapter 2; Chapter 5; exercise: Eldridge *et al.*, 1985; Abraham *et al.*, 2002; state: Sherrey *et al.*, 1988; Pagliardini *et al.*, 2012), and that AE can be produced in preparations with different degrees of reduction (*in vivo*: Chapter 2; Chapter 5; *in situ*: Abdala *et al.*, 2009; anesthetized: Fregosi *et al.*, 1987; Warner *et al.*, 1995; Warner *et al.*, 1992), disinhibition of AE can likely be produced through multiple mechanisms (Feldman *et al.*, 2013).

Bilateral vagotomy also contributed to the disinhibition of both the intercostal muscles and the abdominal muscles, as following bilateral vagotomy, activity in the INT<sub>m</sub> was present under eupneic conditions and activation of the ABS<sub>m</sub> required less CO<sub>2</sub> stimulation (recruited at ~5% CO<sub>2</sub>). The disinhibition by bilateral vagotomy was enough for the intercostal muscles, but not adequate for the complete disinhibition of the abdominal muscles. Interestingly, while both the INT<sub>m</sub> and ABS<sub>m</sub> required disinhibition, the source of this disinhibition was likely different for the two muscle groups (as urethane disinhibited INT<sub>m</sub> activity, but did not disinhibit ABS<sub>m</sub> activity) and the threshold for this inhibition was much lower for the intercostal muscles (2% CO<sub>2</sub> rather than 8% CO<sub>2</sub> when vagi were intact and 0% CO<sub>2</sub> and 5% CO<sub>2</sub> after bilateral vagotomy). As the INT<sub>m</sub> contributed to the inspiratory phase, the primary ventilatory response may have been to increase airflow by increasing breathing frequency or tidal volume through
recruitment of the inspiratory reserve volume. The secondary response of recruiting the ABS\textsubscript{m} appeared to contribute to the additional recruitment of tidal volume, through use of the expiratory reserve volume.

8.5 The role of the Kölliker-Fuse and afferent vagal feedback

The pons and afferent vagal feedback clearly play a role in modifying the breathing pattern. There is evidence that both the pons and afferent vagal feedback control apneas during the breathing cycle, as apneusis (prolonged T\textsubscript{1}) is observed only when both the pons and afferent vagal feedback are eliminated (Stella, 1938). To date, it remains unclear how the pons and afferent vagal feedback contribute to the production of AE. Certainly, when the pons and afferent vagal feedback were intact, AE was recruited with hypercapnia (Chapter 2; Chapter 5). Similarly, when the pons was present but vagal feedback was removed in vivo, AE was again recruited (Lemes and Zoccal, 2014; Chapter 2). Abdala et al. found that complete transection of the pons eliminated the production of AE using a vagotomized in situ preparation (2009). While we are not aware of a study in which AE was produced when the pons had been removed, but vagal feedback remained intact, the data suggest that the pons (or regions within the pons) are critical for the production of AE, and although afferent vagal feedback can modify the threshold of disinhibition required to recruit AE, it is not necessary for the production of AE.

The region(s) within the pons that are critical for the production of AE remain unknown. That being said, a number of areas within the pons have been shown to be critical in generating and modifying the breathing response. The Kölliker-Fuse (KF) and the parabrachial nucleus are often grouped together as critical in regulating the inspiratory off-switch (IOS) (Hilaire et al., 2004; Dutschmann and Dick, 2012). Other areas of the pons, such as the inter-trigeminal region
and the A5/A6 neurons of the locus ceruleus (LC), also play a role in modifying the breathing response by controlling apneas (Chamberlin and Saper, 1998) and modifying the hypoxic and hypercapnic ventilatory response (Guyenet et al., 1993; Teppema et al., 1998), respectively.

The KF inhibited AE by actively exciting the post-inspiratory (post-I) activity, which in turn inhibited augmenting expiratory (aug-E; Molkov et al., 2013) and late expiratory (late-E) abdominal nerve (AbN) phases (Chapter 3). While the KF was not necessary for the generation of AE, it did play a role in modifying the late-E AE activity observed under hypercapnic conditions, acting as a timekeeper for the start of the late-E AE activity (Chapter 3).

Unfortunately, this does not answer the question of what other structures within the pons might be critical for generating AE. It is possible that A5/A6 neurons may play a role in generating AE during the hypercapnic response. Hypercapnia appears to enhance A5 neuron activity more than exposure to hypoxia (Teppema et al., 1998). In addition, chronic intermittent hypoxia, which typically produces AE (Zoccal et al., 2008; Zoccal and Machado, 2010), also enhances A5/A6 neuron activity in rats (Soulier et al., 1997). The appearance of AE during hypercapnia may be dependent on these A5/A6 neurons of the LC generating excitation to the late-E neurons of the pFRG, which might explain, in part, why a complete transection of the pons eliminated AE seen during hypercapnia, but inhibiting the KF alone does not.

Afferent vagal feedback might also play a role in determining the commencement of AE, as this prolonged abdominal activity was observed after chemical inhibition of the KF of a vagotomized in situ preparation (Chapter 3). While the pons and afferent vagal feedback have already been established as a failsafe mechanism for the production of eupnea (Lumsden, 1923; Stella, 1983), it could be possible that both the pons and afferent vagal feedback interact to contribute to the release of AE under hypercapnic conditions.
8.6 Hypercapnia and hypoxia modify the respiratory phases of rodents differently

Exposure to hypercapnia consistently recruits AE in vivo (Sherrey et al., 1988; De Troyer et al., 1989; Abe et al., 1996; O’Halloran et al., 1999; Janczewski et al., 2002; Taccola et al., 2007; Abdala et al., 2009; Chapter 2; Chapter 5), while exposure to hypoxia does not (Fregosi et al., 1987; Smith et al., 1989; Chapter 2; Chapter 6). While there are some species-related differences relating to where AE occurred within the breathing cycle, which will be expanded upon below, there was consistently an increase in tidal volume along with the appearance of AE, suggesting that AE does play a role in enhancing tidal volume by recruiting the expiratory reserve volume (Chapter 5). Based on pulmonary mechanics (Chapter 7), each rodent species studied had an expiratory reserve volume (ERV), which means that AE can indeed be used to recruit the ERV to enhance tidal volume. It raises the question, however, of the importance of AE in species with a small or non-existent ERV. For example, the ribbon seal has a highly compliant chest wall that recoils inward over its entire lung volume, resulting in the residual functional capacity (FRC) that is equal to the residual volume (RV) (Leith, 1976). At the end of a breath (reaching residual functional capacity, FRC), the lungs of the ribbon seal are completely empty, so no ERV is available to the animal (i.e. FRC is equal to the residual volume). Based on the compliance of the respiratory system, it seems unlikely that the ribbon seal would ever need AE, either to help compress the chest wall during expiration or to increase $V_T$. As a result, AE may not ever be used by some species based on the compliance of the system. More research would be needed to determine definitively if ribbon seals produce AE as part of their breathing cycle, which would provide significant information to the relationship between respiratory muscle recruitment and pulmonary mechanics.
The presence of the expiratory abdominal muscle activity during hypercapnia exposure typically presented at the end of the expiratory phase, terminating just before or during the beginning of inspiration, in a late-E phase (gerbil, mice, hamster, spiny rats, rats, pacas, capybara; Chapter 5). Ground squirrels and guinea pigs (Chapter 5) appeared to exploit a similar strategy, but with AE occupying the full expiratory phase, perhaps due to the level of disinhibition or differences in the duration of the breathing cycle phases. Two substantial exceptions to the end-expiratory AE were the agouti (Chapter 5) and the short-tailed opossum (Reilly and White 2009), which exhibited an early-E AE. The reason for this early-E AE remains unclear. Based on pulmonary mechanics data (Chapter 7), the agoutis had the highest total respiratory system compliance and lowest total respiratory system resistance of all the rodents studied (Chapter 7). They also had a substantially high breathing frequency; as a result, agoutis had a passive expiratory time constant ($\tau_E$) that approached the total expiratory duration ($T_E$). This early-E AE may be necessary to decrease the time required to exhale air from the lungs. While little work has investigated the pulmonary mechanics of the short-tailed opossum, Reilly and White (2009) proposed that the early-E AE is necessary for maintaining the high ventilation rate of such a small animal, which may mirror what is occurring in the agoutis.

The evidence of a late-E and early-E AE does raise the question of how the neuronal control of AE allows for the plasticity in the location of AE. As mentioned above, the typical AE response is a late-E abdominal activity generated by the disinhibition of the parafacial respiratory group/retrotrapezoid nucleus (RTN/pFRG; Pagliardini et al., 2011). Although this late-E activity produced by the pFRG under hypercapnic conditions is different from the aug-E activity recorded directly from the BötC (Abdala et al., 2009), there may be evidence of the aug-E neurons contributing to AE. Aug-E neurons appear to have some flexibility in their firing pattern,
as Fortuna et al. showed that in vitro, BötC neurons will transition from an augmenting firing pattern during eupneic conditions to a late-E firing pattern under hypercapnic hypoxic conditions (2008). This proposed expiratory neural plasticity raises the questions: do these aug-E neurons or can other neurons (like the late-E neurons of the pFRG) modify their firing pattern to contribute to the early-E AE observed in the agouti and short-tailed opossum? Or, is this activity from a different source?

An additional (or alternate) possibility is that this early-E AE may be the result of afferent vagal feedback early during expiration. If the presence of an early-E AE is indeed to ensure the lungs are deflating fast enough, then afferent vagal feedback may play a role in this early-E AE. Remmers et al., (1986) found that stimulation of the vagus nerve during the post inspiratory period prolonged the E1 period of post-inspiratory, inspiratory and expiratory neurons, with little change to E2 in cats. While we did not observe a prolonged E1 diaphragm phase, the early-E AE may have been due to a prolonged stimulation of E1 expiratory neurons.

8.7 Strengths and limitations of the thesis research

While this work has highlighted the role of multiple respiratory muscles (the diaphragm, the intercostal muscles, and the abdominal muscles) under progressive hypercapnia and progressive hypoxia, it does not examine all of the respiratory muscles that can contribute to the breathing cycle. There are other respiratory muscles that were not investigated in the study, including a number of laryngeal muscles that contribute to expiratory braking (Dutschmann et al., 2014 for review). It was also difficult to differentiate between the external and intercostal muscles, the abdominal muscles, and the costal or crural diaphragm muscles. As a result of this, even though the work of this thesis is based on a multi-respiratory muscle study, there are still
unanswered questions in how progressive hypercapnia and hypoxia influence all of the respiratory muscles.

A limitation in the comparison of the results from Chapters 1, 2 and 3 is the restriction in the age of each of the preparations used. The in vivo data was completed with adult rats, the in situ work was completed with juvenile rats, and the in vitro work was completed with newborn rats. As a result, every comparison has to be considered based on the age of the preparation used. This is exceptionally true for the in vitro data, in which no AE was ever recorded. This could highlight that central chemoreceptors were not sufficient to recruit AE. Alternatively, the differences in the results might simply be due to the use of a younger preparation, as there was a decrease in the enhancement of $V_T$ between P1 and P8 rats (Stunden et al., 2001; Serra et al., 2001; Wickstrom et al., 2002), so if AE is critical for $V_T$ enhancement, it may not have been seen in younger preparations in vivo or in vitro.

With the comparative studies, there were a number of species with a limited sample size. This small sample size meant that no statistical test could be conducted on the data, limiting the analysis to qualitative measurements. However, this type of experimental design has been used previously (Crosfill and Widdicombe, 1961; Frappell et al., 1992) and while it is limited to a small sample size, the results allow for a substantial comparison between species of a wide range of sizes (gerbil to capybara) and lifestyles (semi-aquatic, burrowers, hibernators, etc.) which revealed variability in breathing requirements.

### 8.8 Future directions

This thesis highlights a number of questions that remain unanswered. The pontine region(s) necessary for the generation of AE have still not been identified. While we suggest a
potential role of the A5/A6 neurons, there are a number of other regions that may contribute to the production of AE. Studies that selectively inhibit certain regions of the pons may help to determine what neural areas are necessary for the production of AE. Additionally, determining if there is any flexibility in the firing activity of the late-E neurons of the pFRG might explain the differences in location of AE among species. In addition, it would be interesting to determine what mechanisms are in the place to result in certain animals, like horses and dogs, exhibiting AE under eupneic conditions. Do horses and dogs simply have a disinhibited pFRG at rest? If so, where is this disinhibition coming from? Another possibility is that this eupneic late-E abdominal activity could be generated from a different area entirely.

8.9 Significance of research

While inspiration is always an active process, expiration is flexible, transitioning from a passive to an active process under certain conditions. Typically, active expiration is not necessary for most mammals under eupneic conditions, yet increasing respiratory drive can recruit AE. Certainly afferent vagal feedback and anesthetic influenced the breathing pattern, however, increasing respiratory drive with hypoxia or hypercapnia typically produced a preliminary increase in breathing frequency and/or tidal volume. These changes were due to a reduction in the inspiratory and/or passive expiratory time, and a recruitment of the inspiratory reserve volume by means of external intercostal muscle activity in addition to the diaphragm activity. While hypoxia did not recruit AE, the increase in tidal volume observed with severe hypercapnia further enhanced tidal volume by activating AE to recruit the expiratory reserve volume. Typically, AE under these conditions occurred at the end of the breathing cycle, as a late-expiratory AE. Species with high breathing frequencies, in addition to a high dynamic
compliance and low resistance, however, recruited AE earlier in the expiratory phase, which may have helped reduce the duration of the expiratory phase ($T_E$). This highlights that AE is also a flexible process, which can influence the breathing response in multiple ways (control $V_T$ vs. control of $T_E$). As the neural control of AE activity was only present after disinhibition of the RTN/pFRG, removal of the inhibitory inputs to the expiratory neural output likely occurred through different mechanisms under different circumstances.

All of the respiratory muscle contractions that power the mammalian breathing pump are under the control of highly organized neurons within the medulla and the pons. These neurons fire to produce the I, E1, E2 and AE breathing phases when necessary. The work of this thesis highlights the role of the coordination of the respiratory muscles under different conditions of increased respiratory drive, and demonstrates the complexity and the multi-functionality of the pontine and medullary centers in controlling the breathing response.
Bibliography


Lumsden, T., 1923. The regulation of respiration. J. Physiol. 58, 81-91.


basal mammalian conditions and eutherian-like tendencies in *Trichosurus*. J. Morphol. 271, 438-450.


Saiki, C., Mirura, A., Furuya, H., Matsumoto S., 2007. Mk-801 alters diaphragmatic activities in
unanesthetized rats differently between normoxic and hypoxia. Life Sciences. 80, 1206-1212.


