POSTNATAL DEVELOPMENT OF CENTRAL RHYTHM
GENERATION OF BREATHING IN MAMMALS

LIENEKE H. MARSHALL
B.Sc. Acadia University, 1998

A THESIS IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS OF THE DEGREE OF
MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES
(Department of Zoology)

THE UNIVERSITY OF BRITISH COLUMBIA
January 2005
© Lieneke Hélène Marshall, 2005
Abstract

The objective of this thesis was to investigate some of the developmental changes that occur in central rhythm generation of breathing in mammals. Specifically, progressive hypothermia was used as a tool to investigate the mechanism of respiratory arrest at very low body temperatures and autoresuscitation upon re-warming. Both in vivo and in vitro techniques were used; the first to identify the point in development when the ability to autoresuscitate from hypothermia-induced respiratory arrest was lost in mammals and the second to determine if the ability of neonatal mammals to autoresuscitate was dependent upon the persistent sodium current (INa_p). Experiments were conducted upon neonates and juveniles of a cold-intolerant species, the rat, and a cold-tolerant species, the hamster.

In vivo experiments revealed that the ability to autoresuscitate was lost in rats between 14 and 20 days after birth and in hamsters between 28 days after birth and adulthood. The time scale over which the ability to autoresuscitate was lost was similar to the time scale over which these animals developed adult-type endothermy and homeothermy. It was hypothesised that the loss of the ability to autoresuscitate over development corresponded to changes in the mechanism of rhythm generation of breathing. Pacemaker properties, primarily dependent upon INa_p, were hypothesised to be the mechanism by which autoresuscitation from hypothermia-induced respiratory arrest occurred. A shift in the relative important of pacemaker properties and network properties of rhythm generation of breathing during the postnatal development of mammals could explain the loss of the ability to autoresuscitate.

In order to examine the role of INa_p-dependent pacemaker properties in respiratory rhythm generation and the phenomenon of autoresuscitation from hypothermia-induced respiratory arrest, riluzole was used to block INa_p in en bloc preparations from neonatal rats and hamsters. Riluzole of two concentrations was bath applied, both at constant temperature and after fictive respiratory arrest at very low temperatures. In neonatal rats, riluzole failed to inhibit rhythm generation and autoresuscitation and instead appeared to block network bursting properties, important for pattern generation. These results are consistent with the hypothesis that INa_p-dependent pacemaker properties are
not essential for rhythm generation in neonatal rats and that respiratory rhythm in this species is instead produced by a heterogenous population of pacemakers including both $I_{Na_p}$-dependent and $I_{Na_p}$-independent cells. In contrast, blockade of $I_{Na_p}$ with riluzole produced fictive respiratory failure in preparations from hamsters via a decline in the frequency of bursting indicating that $I_{Na_p}$ was essential for the generation of respiratory rhythm in this species. Blockade of $I_{Na_p}$ also blocked autoresuscitation entirely in 67% of hamster preparations. These findings are consistent with the hypothesis that $I_{Na_p}$-dependent pacemaker properties are essential for the generation of respiratory rhythm in neonatal hamsters and that these properties are important in the process of autoresuscitation from hypothermia-induced respiratory arrest.
# Table of Contents

Abstract .......................................................................................................................... ii
Table of Contents .......................................................................................................... iv
List of Tables .................................................................................................................. vi
List of Figures ................................................................................................................. vii
Acknowledgements ....................................................................................................... x

Chapter 1: GENERAL INTRODUCTION ...................................................................... 1
  1.1. Hypothermia in neonatal and adult mammals ...................................................... 1
  1.2. Central rhythm generation of breathing ............................................................. 3
  1.3. Overall Objective ............................................................................................... 13

Chapter 2: HYPOTHERMIA-INDUCED RESPIRATORY ARREST AND
  AUTORESUSCITATION IN VIVO .............................................................................. 14
  2.1. Introduction ........................................................................................................ 14
  2.2. Objectives .......................................................................................................... 15
  2.3. Materials and Methods ..................................................................................... 17
  2.4. Results ............................................................................................................... 22
  2.5. Discussion ......................................................................................................... 36
  2.6. Conclusions ...................................................................................................... 47

Chapter 3: THE ROLE OF PERSISTENT SODIUM CURRENTS IN
  RESPIRATORY RHYTHM GENERATION IN VITRO .............................................. 48
  3.1. Introduction ........................................................................................................ 48
  3.2. Objective .......................................................................................................... 54
  3.3. Materials and Methods ..................................................................................... 55
  3.4. Results ............................................................................................................... 63
  3.5. Discussion ......................................................................................................... 84
  3.6. Conclusions ...................................................................................................... 93

Chapter 4: GENERAL DISCUSSION ......................................................................... 95
  4.1. Postnatal development in mammals ................................................................ 95
  4.2. Loss of pacemaker properties of rhythm generation over development .......... 95
  4.3. Development of network properties of rhythm generation ............................ 96
  4.4. Loss of the ability to autoresuscitate over development .................................... 97
  4.5. Roles of pacemaker and network properties of rhythm generation in
    neonates and adults ............................................................................................... 98
4.6. Role of pacemaker properties of rhythm generation in neonates .................. 98
4.7. Species differences between rats and hamsters ......................................... 100
4.8. Developmental differences in rats and hamsters ........................................ 101
4.9. OVERALL CONCLUSIONS ......................................................................... 102
References ........................................................................................................ 104
### List of Tables

**Table 2.1.** Cardiorespiratory variables for P14, P16 and P18-20 rats and P15-20, P21-23 and P26-28 hamsters .................................................................................................................. 26

**Table 2.2.** Tidal volume (\(V_T\)) and inspiratory time (Ti) of the last breath during cooling, the first breath upon re-warming and a breath at the same \(T_B\) during cooling as the first breath upon re-warming ......................................................... 34

**Table 3.1.** Time to the cessation of visible bursting in the absence of riluzole or after administration of riluzole in preparations from P0-4 rats and hamsters ................. 69

**Table 3.2.** The effect of riluzole on the fictive respiratory output at 27°C (including burst frequency, amplitude, duration and area) in preparations from P0-2 and P3-4 rats and hamsters ........................................................................ 72

**Table 3.3.** Actual time corresponding to \(T_R\), where \(T_R=0\) was just prior to administration of riluzole and \(T_R=1.0\) was when bursting ceased in preparations from P0-2 and P3-4 rats .................................................................................................................. 75

**Table 3.4.** Actual time corresponding to \(T_R\), where \(T_R=0\) was just prior to administration of riluzole and \(T_R=1.0\) is when bursting ceased in preparations from P0-2 and P3-4 hamsters .................................................................................................................. 75

**Table 3.5.** The effect of age and species on the frequency, amplitude, duration and area of fictive respiratory output prior to cooling and after re-warming in *en bloc* preparations from P0-2 and P3-4 rats and hamsters ................................................................. 76

**Table 3.6.** The effect of age on temperature of fictive respiratory arrest and autoresuscitation upon re-warming in preparations from P0-2 and P3-4 rats and hamsters .................................................................................................................. 76

**Table 3.7.** The frequency, amplitude, duration and area of fictive respiratory output prior to cooling and after re-warming of *en bloc* preparations from P0-2 rats and hamsters that were either bathed in riluzole or not bathed in riluzole prior to re-warming .................................................................................................................. 81

**Table 3.8.** The temperature of fictive respiratory arrest and autoresuscitation upon re-warming of preparations from P0-2 rats and hamsters including those bathed in riluzole and those not bathed in riluzole prior to re-warming ....... 81
List of Figures

Figure 1.1. Dorsal view of rat brainstem and cervical spinal cord indicating the regions important in the generation of breathing...........................................4

Figure 1.2. Parasagittal view of rat rostral medulla and caudal cerebellum showing the location of the preBötzinger complex and parafacial respiratory group.........................................................4

Figure 1.3. The three primary types of models of central generation of breathing. A) network model B) pacemaker model and C) hybrid pacemaker-network model .................................................................6

Figure 1.4. Current-voltage relationship typical of voltage-dependent pacemaker neurons........................................................................................................8

Figure 2.1. Apparatus for in vivo experiments..............................................................18

Figure 2.2. Schematic of how respiratory variables were determined.............................21

Figure 2.3. Time course of change in mean $T_b$ during progressive hypothermia and subsequent re-warming to 35°C for A) rats aged 14, 16, 18-20 days and B) hamsters aged 15-20, 21-23 and 26-28 days.........................................................27

Figure 2.4. Breathing frequency during cooling and re-warming in A) P14 B) P16 C) P18-20 rats..............................................................................28

Figure 2.5. Breathing frequency during cooling and re-warming in A) P15-20 B) P21-23 C) P26-28 hamsters .................................................................29

Figure 2.6. Heart rate during cooling and re-warming in A) P14 B) P16 C) P18-20 rats..............................................................................30

Figure 2.7. Heart rate during cooling and re-warming in A) P15-20 B) P21-23 C) P26-28 hamsters..............................................................................31
Figure 2.8. $T_B$ of last breath before respiratory arrest and first breath after respiratory arrest (autoresuscitation) for A) rats aged 14, 16, 18-20 days and B) hamsters aged 15-20, 21-23 and 26-28 days.................................32

Figure 2.9. The relationship between age and % survival of A) rats aged 14, 16, 18-20 days and B) hamsters aged 15-20, 21-23 and 26-28 days.................................33

Figure 2.10. Tidal volume ($V_t$) and inspiratory time ($T_i$) of the last breath during cooling, the first breath upon re-warming and a breath at the same $T_B$ during cooling as the first breath upon re-warming for A) rats aged 14-20 days and B) hamsters aged 21-28 days .........................................................34

Figure 3.1. Respiratory-like motor discharge patterns generated by hamster brainstem-spinal cord preparations at 27°C including A) incrementing or augmenting pattern, B) bell-shaped pattern and C) decrementing pattern ........50

Figure 3.2. Possible roles of ionic channels in the generation of bursting activity in an inspiratory pacemaker neuron.................................................................52

Figure 3.3. In vitro experimental set-up ..............................................................57

Figure 3.4. Sample recordings showing the effect of riluzole on motor output of brainstem-spinal cords from P0-4 rats and hamsters at 27°C.................................70

Figure 3.5. The effect of two concentrations of riluzole on A) the frequency and B) amplitude of fictive breathing in brainstem-spinal cord preparations from P0-4 rats at constant temperature (27°C) .........................................................71

Figure 3.6. The effect of two concentrations of riluzole on A) the frequency and B) amplitude of fictive breathing in brainstem-spinal cord preparation from P0-4 hamsters at constant temperature (27°C).........................................................71

Figure 3.7. The effect of riluzole (200 $\mu$M) on A) fictive breathing frequency B) burst amplitude C) duration and D) area in P0-2 and P3-4 rats at constant temperature (27°C) ....................................................................73

Figure 3.8. The effect of riluzole (200 $\mu$M) on A) fictive breathing frequency B) burst amplitude C) duration and D) area in P0-2 and P3-4 hamsters at constant temperature (27°C) ....................................................................74
Figure 3.9. The effect of transitional cooling and re-warming on fictive breathing frequency in brainstem-spinal cord preparations of A) rats and B) hamsters aged P0-2 and P3-4..........................77

Figure 3.10. The effect of transitional cooling and re-warming on burst amplitude, duration and area in brainstem-spinal cord preparations of A) rats and B) hamsters aged P0-2 and P3-4 ..................................................78

Figure 3.11. Sample recordings of motor output from brainstem-spinal cord preparations of rats (P0-2) showing A) the effect of transitional cooling and re-warming on motor output and B) the effect of riluzole on the ability to autoresuscitate from hypothermia-induced fictive respiratory arrest ...............79

Figure 3.12. Sample recordings of motor output from brainstem-spinal cord preparations of hamsters (P0-2) showing the effect of transitional cooling and re-warming on motor output and the effect of riluzole on the ability to autoresuscitate from hypothermia-induced fictive respiratory arrest ...........80

Figure 3.13. The effect of transitional cooling and re-warming on fictive breathing frequency in brainstem-spinal cord preparations of A) P0-2 rats and B) P0-2 hamsters that were either bathed in riluzole or not bathed in riluzole prior to re-warming ..........................................................82

Figure 3.14. The effect of transitional cooling and re-warming on burst amplitude, duration and area in brainstem-spinal cord preparations of A) P0-2 rats and B) P0-2 hamsters that were either bathed in riluzole or not bathed in riluzole prior to re-warming .......................................................83
Acknowledgements

I would like to thank everyone who contributed to the completion of this thesis. Thank you to my supervisor, Bill Milsom, for your guidance, patience and encouragement throughout this project. You are a fabulous mentor and an excellent example. Thank you also to my supervisory committee, Colin Brauner and Vanessa Auld, for your advice and support during the planning and final stages of the completion of this thesis.

Thank you to the Milsom Lab, both past and present members, for your support and assistance over the last couple of years. Particularly, I would like to thank Angie O’Neill for her unquenchable positive attitude, friendship and sense of humour, Charissa Fung for her generosity and for always being ready with a hug and Andrea Corcoran for being a great office-mate and for her help on many occasions during the data collection phase of this work.

Thank you also to my wonderful family and friends for your love and your faith in me. Particularly, I would like to thank James Pegg, for his patience, love and understanding, without which this project would have been both more difficult and less fun, and Roberta Cottam for her excellent friendship and continuing support throughout all of my endeavours.

Last but not least, thank you to the staff of the Department of Zoology and the Animal Care Centre for their technical and administrative assistance throughout this project.
1. INTRODUCTION

1.1. HYPOTHERMIA IN NEONATAL AND ADULT MAMMALS

Progressive hypothermia is associated with a sequence of events, including failure of thermogenesis followed by respiratory arrest and cardiac arrest that results in death for most mammals. During this progression, breathing and circulation remain effective at meeting the metabolic needs of the animal up until the point at which respiratory and cardiac arrest occur at very low body temperatures. (Adolph, 1948b; Rosenhain & Penrod, 1951; Osborne & Milsom, 1993). Neonatal mammals are generally more tolerant of hypothermia in that they undergo respiratory arrest at lower body temperatures (Te), and also die at lower Te, than adults of the same species (Adolph, 1948b). It also appears that the hearts of neonates, unlike those of adults, can continue to beat long after respiratory arrest has occurred (Hill, 2000). Since metabolic rate is profoundly reduced at such low Te, neonatal mammals with no active ventilation of the lungs can still exchange sufficient gases to meet their metabolic needs by passive respiration through an open glottis (Hill, 2000).

1.1.1. Autoresuscitation

If neonates of many mammal species (eg. rats and hamsters) are artificially re-warmed from extreme hypothermia they spontaneously re-start their breathing during the re-warming process, a phenomenon known as autoresuscitation. Adolph (1951) found that neonatal rats and hamsters could survive up to 3 hours of respiratory arrest at body temperatures below 5°C. When they were slowly re-warmed from below 5°C to ~10°C, neonates re-started their breathing and when re-warmed to a normal Te of 35° exhibited no negative effects whatsoever (Adolph, 1951; Hill, 2000; Tattersall & Milsom, 2003). This is in sharp contrast to what is observed in adult mammals. Once respiratory arrest has occurred in adult mammals, artificial re-warming from hypothermia will not restore normal breathing. Adult mammals fail to autoresuscitate from hypothermia-induced respiratory arrest and subsequently die as they are re-warmed (Adolph, 1948a).
1.1.2. Mechanism of hypothermia-induced respiratory arrest

A detailed analysis of respiratory arrest in hypothermia provides interesting insights into the mechanism of central respiratory rhythm generation in mammals. Considerable evidence has been collected to suggest that in vivo hypothermia-induced respiratory arrest occurs as a result of the failure of the central rhythm generator (CRG) for breathing. When intact mammals are gradually cooled, the frequency of breathing (fR) decreases progressively with Tb while the tidal volume (VT) of each breath remains constant or is slightly elevated (Corcoran & Milsom, 2003; Tattersall & Milsom, 2003). When respiratory arrest occurs, the VT of the last breath before arrest is not significantly different than that of a breath at 35-37°C (Corcoran & Milsom, 2003; Tattersall & Milsom, 2003). The decline in fR, accompanied by no decline in VT, implies that ventilation in hypothermia fails at the level of the CRG and not at the level of downstream elements, such as the pre-motor and motor neurons.

Respiratory arrest and autoresuscitation can also occur in vitro using preparations that generate respiratory-like motor discharge known as “fictive breathing”. Mellen et al. (2002) progressively cooled and re-warmed fictively breathing sagittal slice preparations from neonatal rats. Recordings were made simultaneously from the surface of the brainstem in a region believed to contain the CRG for breathing and from the motor nerve that innervates the diaphragm in vivo (the phrenic nerve root). The magnitude of the phrenic nerve discharge, representative of VT in a living animal, did not change significantly during cooling and re-warming while the frequency of discharge from both locations decreased with temperature until fictive respiratory arrest occurred. Fictive respiratory arrest occurred abruptly as the discharge from the preBotzinger complex and the phrenic nerve ceased simultaneously, indicating a sudden failure of the CRG at low temperatures. In addition, Milsom et al. (2002) found that, while the fictive respiratory frequency of transverse brainstem slices was temperature sensitive, the amplitude and duration of the motor output, representing VT, was not. Thus, hypothermia causes a progressive decrease in breathing frequency without a significant change in the size of each breath indicating that the CRG of mammals is temperature sensitive whereas the individual pattern generating elements are largely unaffected by hypothermia.
Intact neonates, and in vitro preparations from neonatal mammals, are capable of autoresuscitation from hypothermia-induced respiratory arrest if they are re-warmed. Since neonatal mammals are capable of autoresuscitation from hypothermia-induced respiratory arrest, the CRG of neonates must be able to spontaneously re-start in these animals (Milsom et al., 2002). In contrast, the CRG of adults cannot re-start once it has been stopped, even if the cause of respiratory arrest is removed (i.e., the animal is artificially re-warmed from hypothermia). Therefore the critical difference between neonates that are able to autoresuscitate and adults that are not able to autoresuscitate must reside within the CRG for breathing.

1.2. CENTRAL RHYTHM GENERATION OF BREATHING

Breathing in mammals is generated in the brainstem. Neurons with respiratory related activity, form a ventrolateral column known as the ventral respiratory group (VRG) that extends caudally from the facial nucleus (VII) through the medulla to the spinal cord (Figure 1.1). A region known as the preBötzing complex, located at the rostral end of the VRG, has been identified as the location of the primary CRG for breathing (Figure 1.2; Rekling & Feldman, 1998; Feldman et al., 2003). Slices made from the medulla that include only the preBötzing complex are capable of generating respiratory rhythm (Smith, 1991). If the preBötzing complex is removed from the rest of the medulla either by transection, kainic acid injections or by way of neurokinin-1 antagonists specific for most preBötzing complex neurons, respiratory rhythm is either severely impaired or completely abolished (Smith, 1991; Gray et al., 1999; Gray et al., 2001; Guyenet & Wang, 2001; Mutolo et al., 2002). Thus the preBötzing complex is both necessary and sufficient for the generation of breathing. The preBötzing complex contains all the neuron types required for the generation of breathing according to most models of central rhythm generation (Feldman, 1986; Richter & Sprey, 2001). The cells in this region are predominantly propriobulbar, that is they synapse with other cells in the medulla, and include phase spanning neurons (important for the transition between inspiratory and expiratory activity) as well as pacemaker cells with voltage dependent endogenous rhythms (Smith, 1991; Funk & Feldman, 1995; Ramirez & Richter, 1996). Finally, the preBötzing complex has been found to be capable of generating a number of different respiratory rhythms as a function of the level of oxygenation. A eupnoea-like
Figure 1.1. Dorsal view of rat brainstem and cervical spinal cord indicating the regions important in the generation of breathing. Abbreviations include: A5, pontine A5 group; RTN, retrotrapezoid nucleus; Bötc, Bötzinger complex; preBötc, preBötzinger complex; rVRG, rostral ventral respiratory group; NTS, nucleus tractus solitarius; cVRG, caudal ventral respiratory group. Modified from: Rekling & Feldman (1998).

Figure 1.2. Parasagittal view of rat rostral medulla and caudal cerebellum showing the location of the preBötzinger complex (preBötc) and parafacial respiratory group (pre-I). Other abbreviations include RTN, retrotrapezoid nucleus; VII, facial nucleus; cNA, compact division of the nucleus ambiguus; Bötc, Bötzinger complex; NA, nucleus ambiguus; rVRG, rostral ventral respiratory group; LRN, lateral reticular formation. Redrawn from: Feldman et al. (2003).
breathing pattern, accompanied by occasional augmented breaths, are produced in normoxia and a gasp-like pattern is generated in hypoxia (Lieske et al., 2000). This recent finding explains conflicting observations in the past in which lesions in the region of the preBötzinger complex blocked eupnea, gasping or both (Ramirez et al., 1998; Koshiya & Smith, 1999).

Another region of the brainstem has recently become of interest to the study of central rhythm generation of breathing. This region is known as the “parafacial respiratory group” (pFRG) because of its location just ventrolateral to the facial nucleus, and also the “pre-l” area, because the neurons in this region fire ~500 milliseconds before inspiratory activity in the preBötzinger complex occurs (Figure 1.2). Since the neurons in the pFRG fire before neurons in the preBötzinger complex this suggests that the pFRG may be the site of the primary rhythm generator. It may be, however, that the pre-l neurons in the pFRG receive excitatory inputs from neurons in other areas of the brainstem before firing or that the preBötzinger complex and pFRG act together to produce rhythm as part of a coupled oscillator (Feldman et al., 2003; Onimaru & Homma, 2003; Duffin, 2004).

1.2.1. Models of central rhythm generation
While most researchers now agree that the preBötzinger complex is both necessary and sufficient for the generation of breathing in mammals there is still considerable controversy about the mechanism of central rhythm generation. It may be that the root cause of the controversy is the great diversity of preparations used in the study of central rhythm generation. It is likely that the mechanism of respiratory rhythm generation is different in reduced preparations and intact animals. While not often considered, it is likely that postnatal age of the animals used in the various preparations also plays a role in the controversy. For practical reasons, studies using reduced preparations use tissue collected from neonates whereas studies using intact animals typically use adults. Observations made using the different preparations of animals of different postnatal ages have led to the proposal of many models of central rhythm generation, each of which fits the observations made using a particular preparation. There are currently three groups of models of central rhythm generation; A) network models, in which rhythm is produced by the interactions between different cells or cell
types B) pacemaker models, in which rhythm is produced by the intrinsic bursting of pacemaker cells and C) hybrid models, in which rhythm is produced by a combination of both network and pacemaker properties (Figure 1.3).

Figure 1.3. The three primary types of models of central generation of breathing. A) network model B) pacemaker model and C) hybrid pacemaker-network model. Redrawn from: Funk and Feldman (1995) and Smith et al. (2000).
In these models, rhythm generating and pattern generating circuits are often described as conceptually separate processes. Indeed, there is considerable empirical evidence to indicate that the mechanisms that generate the rhythm of breathing are different from those that generate the pattern of each breath (Feldman, 1986; Duffin, 2004; Smith et al., 2004). However, this hypothesis remains a controversial one since in some situations an apparent overlap in the circuitry generating respiratory rhythm and respiratory pattern has been reported (McCrimmon et al., 2000; Ramirez et al., 2002).

A) Network models

There have been many network models of central rhythm generation put forward over the last decade, most of them designed using data collected in *in vivo* investigations of rhythm generation in adult mammals (Duffin et al., 1995; Feldman & Smith, 1995). These models of central rhythm generation are based on reciprocal inhibition in which various respiratory-related neurons inhibit one another through γ-aminobutyric acid (GABA) and glycine mediated mechanisms (Figure 1.3A). Reciprocal inhibition occurs when two or more neurons or neuron groups are connected by inhibitory synapses with the result that when one neuron group fires it inhibits the activity of the other neuron groups. For instance, during early inspiration, Early I neurons actively inhibit Late I neurons, Early E neurons and E neurons. During early expiration, Early E neurons actively inhibit Early I, I, Late I and E neurons. Reciprocal inhibition, and the oscillations in activity that result from this mechanism, can explain both rhythmogenesis and the co-ordination of the inspiratory and expiratory phases of breathing (Funk & Feldman, 1995; Hilaire & Pasaro, 2003).

Considerable evidence has been collected in support of network models of central rhythm generation. Since according to network models, rhythm generation is proposed to depend upon the synaptic connections between neurons, blockade of synaptic transmission via glycinergic and/ or GABAergic mechanisms should block rhythm generation. Indeed, many authors have demonstrated that blockade of glycine and/ or GABA, both of which are involved in reciprocal inhibition, abolishes respiratory rhythm generation in adult mammals *in vivo* (Paton et al., 1994; Paton & Richter, 1995; Pierrefiche et al., 1998). If synaptic transmission is blocked instead by removal of chloride ions the result is the same; rhythm generation is completely blocked in arterially
perfused adult rats (Hayashi & Lipski, 1992). Thus, these authors conclude that inhibitory synaptic transmission is required for rhythm generation, providing support for a network model.

**B) Pacemaker models**

Pacemaker models of rhythm generation arose out of two principal discoveries. The first is that the preBötzinger complex, hypothesised to be the location of the kernel of the CRG, contains cells with pacemaker properties. The second is that, at least in *in vitro* preparations from neonatal mammals, blockade of inhibitory synaptic interactions (glycinergic and GABAergic) does not significantly alter the rhythm generated by the CRG (Johnson *et al.*, 1994; Ramirez & Richter, 1996; Shao & Feldman, 1997; Brockhaus & Ballanyi, 1998). As a result, numerous hypotheses have been proposed in which the primary rhythm generator for breathing consists of a group of “pacemaker” neurons with voltage dependent endogenous rhythms (Figure 1.3B) (Smith, 1991; Butera *et al.*, 1999a; Butera *et al.*, 1999b; Rybak *et al.*, 2003).

**Figure 1.4.** Current-voltage relationship typical of voltage-dependent pacemaker neurons; this cell generates rhythmic bursting between membrane potentials of −55 mV and −45 mV, is quiescent at membrane potentials below −55 mV and generates tonic discharge at membrane potentials above −45 mV.
The pacemaker cells identified in the preBötzinger complex generate rhythmic activity in a voltage dependent manner as a result of intrinsic membrane conductances. These pacemakers produce oscillatory discharges between membrane potentials of \(-45\) mV and \(-55\) mV, along the downward slope of the current-voltage curve (Figure 1.4). Below this voltage range the cells are inactive (quiescent) and above this voltage range they are tonically active (Feldman & Smith, 1995; Rekling & Feldman, 1998).

Pacemaker cells have been identified that fire prior to inspiratory activity and also during inspiratory activity (Johnson et al., 1994; Onimaru et al., 1995; Koshiya & Smith, 1999). Cells in the preBötzinger complex with pacemaker properties appear to be coupled together via excitatory synaptic connections resulting in the synchronous bursting activity hypothesised to produce the rhythm of breathing (Rekling & Feldman, 1998; Koshiya & Smith, 1999). The intrinsic rhythms of these pacemaker cells are believed to be primarily dependent on the persistent sodium current (\(I_{Na_p}\)), however there is some evidence to indicate that the delayed rectifier potassium current may play an accessory role in the generation of pacemaker activity (Butera et al., 1999a; Del Negro et al., 2002; Rybak et al., 2003). When persistent sodium currents are blocked the endogenous rhythms of most pacemaker cells in the preBötzinger complex are abolished (Del Negro et al., 2002; Rybak et al., 2003). However, a small sub-population of pacemaker cells in the preBötzinger complex produce rhythm independent of \(I_{Na_p}\) and instead appear to be dependent upon calcium-mediated mechanisms for pacemaking behaviour (Pena et al., 2004).

There is considerable evidence collected to indicate that the endogenous rhythms of pacemaker cells are sufficient for the generation of respiratory rhythm, at least in neonatal in vitro preparations, however there is little evidence to indicate that these same properties are necessary for rhythm to be produced. As discussed above, if \(I_{Na_p}\) is blocked in pacemaker cells these cells stop producing rhythmic discharge. However, both Del Negro et al. (2002) and Parkis (2002) found that blockade of persistent sodium currents did not profoundly affect the rhythm of motor output. This led to the conclusion that pacemaking is an emergent network property rather than the essential “kernel” of the CRG (Del Negro et al., 2002; Feldman et al., 2003). Since the discovery of \(I_{Na_p}\)-independent pacemaker cells in the preBötzinger complex, in addition to the already
identified \(\text{INa}_p\)-dependent pacemakers, this conclusion should be questioned. It may be that sufficient pacemaker cells remained active in these experiments, despite blockade of \(\text{INa}_p\), to produce normal rhythmic motor discharge (Pena et al., 2004).

C) Hybrid pacemaker-network model
In 1995, Smith et al. put forward a hybrid pacemaker-network model that attempted to unify aspects from both network and pacemaker models of central rhythm generation (Figure 1.3C). Since then, the model has been modified and elaborated upon by many authors, based on both computer modelling and experimental results (Butera et al., 1999a; Butera et al., 1999b; Smith et al., 2000; Del Negro et al., 2001). According to this model, the disparity between observations made using \textit{in vitro} techniques and those made using \textit{in vivo} techniques is a result of the increasing complexity of the rhythm generating system as one moves from reduced preparations to the intact animal. While the model arose first as a way to interpret results from different preparations, regardless of developmental age, it developed into a model that adequately explained the differences in rhythm generation observed between neonatal and adult mammals.

The hybrid pacemaker-network model is based upon three assumptions (Smith et al., 2000). The first is that there is a kernel of respiratory rhythm generating neurons located in the preBötzinger complex and that this kernel remains functional throughout the development of mammals. The second is that the pacemaking properties of the central kernel directly produce respiratory rhythm in neonatal mammals. The third is that, while the pacemakers may continue to generate rhythm throughout development, a network dependent upon reciprocal inhibition, regulates the rhythm they produce in the adult mammal. Thus, the most basic of rhythm generating mechanisms, pacemaking, produces breathing in neonates but with development the basic mechanism is overlayed with inhibitory mechanisms that depend on synaptic relationships rather than on endogenous rhythms. It is these inhibitory mechanisms that give rise to the synchronous oscillations of inspiratory and expiratory activities that make up the complete respiratory pattern (Smith et al., 2000).

The “kernel” of the CRG is hypothesised to consist of a heterogenous group of
pacemaker cells connected by excitatory synapses (Figure 1.3C). This pacemaking kernel displays synchronous bursting that arises from a voltage activated persistent sodium current. There is, as described earlier, considerable evidence to indicate that pacemaker properties alone are sufficient for rhythm generation in in vitro neonatal systems (Shao & Feldman, 1997; Brockhaus & Ballanyi, 1998; Rekling & Feldman, 1998). The role of pacemaker cells in rhythm generation in vivo and in adult or juvenile mammal systems is much less certain. There are significant technical difficulties involved in blocking synaptic inhibition entirely in adults in vivo and therefore it is difficult to determine if pacemaker properties are adequate for respiratory rhythm generation in adult mammals (Smith et al., 2000). However, there are numerous studies that indicate that network properties of rhythm generation, such as synaptic inhibition via GABAergic and glycinergic mechanisms are of primary importance to normal rhythm generation in adult mammals (Paton et al., 1994; Paton & Richter, 1995; Pierrefiche et al., 1998). Thus, in the neonate the CRG appears to be primarily dependent upon pacemaker properties whereas in the adult the CRG appears to be primarily dependent upon network properties but may also be dependent upon pacemaker properties.

1.2.2. Development of the CRG: the maturational network-burster model

The hybrid model of central respiratory rhythm generation offers a hypothetical design of the CRG that adequately explains the differential rhythm generation observed in adult and neonatal mammals. The maturational network-burster model builds upon the hybrid pacemaker-network model of rhythm generation in mammals and provides a mechanistic answer to how the CRG develops from primarily pacemaker-driven in neonates to primarily network-driven in adults (Richter & Spyer, 2001). As mammals develop and neurons mature the resting membrane potential of respiratory-related neurons becomes progressively more negative (hyperpolarized). Thus, the resting membrane potential of respiratory neurons (including pacemaker neurons) in neonatal rodents is between −45 mV and −50 mV, significantly higher than that of fully mature neurons which is −65 mV (Richter & Spyer, 2001). The resting membrane potential of immature neurons is well above the threshold determined for many voltage-dependent channels, including the persistent sodium channel. Therefore the intrinsic ion conductances required for the endogenous bursting of respiratory pacemaker neurons are present in the CRG of neonates. As the resting membrane potential of the neurons
becomes more negative over development, these voltage-dependent channels are inactivated and endogenous bursting of the cells stops (Richter & Spyer, 2001). Thus, according to the maturational network-burster model, endogenous bursting behaviour does not occur independent of excitatory synaptic connections in the adult CRG (Richter & Spyer, 2001).

1.2.3. 

Models of central rhythm generation and autoresuscitation
Together, the hybrid model and maturational network-burster model suggest a change in the CRG of mammals that could underlie the loss of the ability to autoresuscitate from hypothermia-induced respiratory arrest as mammals develop. According to these models, the CRG of neonates is primarily dependent upon the endogenous bursting of pacemaker cells which continue to generate bursting, and therefore breathing, as long as the resting membrane potential of the respiratory neurons is between $-45 \text{ mV}$ and $-50 \text{ mV}$ (Smith et al., 2000; Richter & Spyer, 2001). In severe hypothermia the CRG of neonates arrests and breathing stops (Mellen et al., 2002; Milsom et al., 2002). Upon re-warming, endogenous bursting could resume since the membrane potential would likely be within the active voltage range of the relevant channels. In contrast, the CRG of adults is much more dependent upon network properties of rhythm generation than the CRG of neonates. According to the model put forward by Richter & Spyer (2001), endogenous bursting of pacemaker cells likely does not occur in the mature respiratory network because the resting membrane potential of the respiratory neurons is outside the active range for the various ion conductances required for this type of activity. If respiratory arrest occurred in hypothermia the membrane potential of the respiratory neurons of adults would likely rest at $-65 \text{ mV}$. At this membrane potential none of the intrinsic ion conductances required for endogenous bursting are active. Since network properties of rhythm generation, such as reciprocal inhibition, depend upon oscillations in activity between two neurons or neuron groups, if neither is active the oscillations that normally produce breathing are absent (Shao & Feldman, 1997). Because, according to the maturational network-burster model, endogenous bursting is also absent, the mature CRG is unlikely to be able to re-start if re-warmed from severe hypothermia.
1.3. **OVERALL OBJECTIVE**

The objective of this thesis was to investigate some of the developmental changes that occur in central rhythm generation of breathing in mammals. Specifically, progressive hypothermia was used as a tool to investigate the mechanism of respiratory arrest at very low body temperatures and autoresuscitation upon re-warming. Both *in vivo* and *in vitro* techniques were used; the first to identify the point in development when the ability to autoresuscitate from hypothermia-induced respiratory arrest was lost and the second to determine if the ability of neonatal mammals to autoresuscitate was dependent upon the persistent sodium current. Experiments were conducted upon neonates and juveniles of a cold-intolerant species, the rat, and a cold-tolerant species, the hamster. We hypothesised that between the ages of 12 and 28 days after birth, juvenile rats and hamsters would lose the ability to autoresuscitate from hypothermia-induced respiratory arrest indicating a change in the mechanism of central rhythm generation of breathing at this age. We also hypothesised that the persistent sodium current would be essential for rhythm generation of breathing in neonatal rats and hamsters and that blockade of this current would inhibit autoresuscitation from hypothermia-induced respiratory arrest in these animals.
2. HYPOTHERMIA-INDUCED RESPIRATORY ARREST AND AUTORESUSCITATION IN VIVO

2.1. INTRODUCTION

Hypothermia is defined as an unregulated decline in body temperature (TB). In most mammals it is a pathological state characterised by an initial thermogenic effort, accompanied by an increase in metabolic rate. As TB continues to fall thermogenesis fails, followed by respiratory arrest, cardiac arrest and finally death. Some mammals, however, tolerate extremely low TB and suffer no negative effects, notably very young individuals and members of hibernating species. For these mammals, very low TB may not be pathological. In fact, hibernating mammals utilise low TB to maintain very low metabolic rates in order to conserve energy at times of reduced energy availability.

If neonates of some mammal species (eg. rats and hamsters) are artificially re-warmed from extreme hypothermia they spontaneously re-start their breathing during the re-warming process, a phenomenon known as autoresuscitation (Adolph, 1951; Hill, 2000; Tattersall & Milsom, 2003). However, once respiratory arrest has occurred in adult mammals, artificial re-warming from hypothermia will not restore normal breathing. Adult mammals fail to autoresuscitate from respiratory arrest and subsequently die as they are warmed (Adolph, 1948a). It appears that this difference between neonatal and adult mammals is due to a change in the mechanism underlying respiratory rhythm generation (Mellen et al., 2002; Milsom et al., 2002). In the following experiments we attempt to determine “the window” in development during which the ability to autoresuscitate is lost, indicating a change in the central rhythm generator (CRG) for breathing at this age.

As mentioned above, neonatal mammals are significantly more tolerant of hypothermia than adults. This difference is most pronounced in species with altricial young (ie. those young born in a comparatively undeveloped state; Adolph, 1951). Altricial neonates have very limited thermogenic abilities. Rats of around 2 days of age are capable of some brown adipose tissue (BAT) thermogenesis but no shivering thermogenesis.
whereas hamsters are incapable of any thermogenesis until around postnatal day 12 (Blumberg et al., 1997; Blumberg & Sokoloff, 1998). Neither species is able to maintain TB in room temperature air until 15-18 days of age (Blumberg & Sokoloff, 1998; Sokoloff et al., 2000). Neonatal rats, for example have been demonstrated to tolerate TB as low as 5°C for up to 3 hours while adults can tolerate TB of ~15°C for only a short while before death occurs. It has been proposed that the loss of neonatal cold tolerance over development occurs gradually over a similar time scale as the development of endothermy. In the following experiments we attempt to provide a time scale for the loss of neonatal cold tolerance, as measured by the TB of respiratory arrest, and to determine if this developmental change is linked to the development of endothermy and the loss of the ability to autoresuscitate from hypothermia-induced respiratory arrest.

Finally, gasping is known to play a role in autoresuscitation from hypoxia-induced respiratory arrest in both neonatal and adult mammals of many different species. However, it has been demonstrated that gasping does not occur either in extreme hypothermia, or as part of autoresuscitation from hypothermia induced respiratory arrest, in neonatal rats or hamsters (Corcoran & Milsom, 2003; Tattersall & Milsom, 2003). These observations concur with those of Hill (2000) who found no evidence to indicate that neonatal mammals suffered from hypoxia or anoxia during hypothermia-induced respiratory arrest. Very young neonatal mammals are capable of meeting their oxygen needs in hypothermia through passive gas exchange through the open glottis as long as the heart continues to beat (Hill, 2000). During development, both body size and oxygen demands increase. Older mammals may therefore suffer hypoxia during hypothermia-induced respiratory arrest and may gasp during re-warming. To determine if older mammals suffered from hypoxia during hypothermia-induced respiratory arrest we examined the role of gasping in the process of autoresuscitation in rats and hamsters as a function of age.

2.2. OBJECTIVES

The primary objective of these in vivo experiments was to determine at what point in development rats and hamsters lost the ability to autoresuscitate. Other objectives included determining if there were age-related changes in the cardiorespiratory
responses to hypothermia and the role of gasping in recovery from hypothermic respiratory arrest in juvenile mammals.
2.3. MATERIALS AND METHODS

2.3.1. Animals
Experiments were performed on 12 juvenile Sprague Dawley rats (*Rattus norvegicus*), aged 14-20 days and 18 juvenile Syrian hamsters (*Mesocricetus auratus*), aged 15-28 days. Animals were grouped by postnatal age (in days after birth) into three groups for each species. Rats were grouped by postnatal age (P) into P14, P16 and P18-20 groups. Hamsters were grouped into P15-20, P21-23 and P26-28 age groups. All experiments were completed with the prior approval of the University of British Columbia Animal Care Committee acting under the guidelines of the Canadian Council for Animal Care. In the case of rats, the animals were provided by the University of British Columbia Animal Care Centre and delivered to the laboratory the morning of the experiment. In the case of hamsters, timed pregnant females (gestation day 8-12) were obtained from a commercial supplier (Charles River Laboratories, Canada) and maintained until delivery in the lab. Pregnant females were housed separately and provided with water and rodent chow *ad libitum*, supplemented with sunflower seeds and fruit. The exact time of birth was noted for accurate determination of the age of the neonates. Individual neonates were removed from the litter as needed for experiments.

2.3.2. Animal instrumentation and equipment
Neonatal rats and hamsters were removed from the mother’s cage immediately prior to the experiment. The animal was weighed and then lightly anaesthetised in a chamber with Halothane (1-3 %) for 1-2 minutes. After removal to the surgery area, the animal was shaved on the ventral, dorsal and lateral body surfaces to minimise insulation, and therefore thermoregulatory defence during cooling, and insure a similar rate of cooling of animals at different developmental stages. Xylocaine (0.1 mL of 2%) was injected under the skin on both sides of the chest wall just caudal to the forelimbs. Two small incisions were made in the skin, one at the site of each injection, and a hollow was made between the skin layers and the chest wall musculature with blunt forceps. Disk electrodes (diameter 3-4 mm), for measuring the electrocardiogram (ECG) and respiratory impedance, were inserted between the skin and muscle layers on each side of the chest wall and the incisions sealed with tissue glue (Periacryl). The wires leading from the electrodes (7 strand stainless steel coated with silicon) were passed under the
forelimbs and secured together on the back with a piece of medical tape. At this point the electrodes were tested to insure that each was placed correctly to receive good signals. Next, a Physitemp (Model BAT-12, Type T) thermocouple, coated in flexible plastic tubing was inserted into the rectum to a depth of 1.5 cm and secured to the tail with medical tape. Once the animal was instrumented it was placed into the temperature control chamber (a water-jacketed, air-filled cylinder; Figure 2.1) and the leads from the electrodes and thermocouple pulled through a port in the top of the chamber. The temperature of the water flowing through the walls of the cylinder was regulated with a Lauda refrigerating circulator (RC-6) filled with a mixture of 50% ethylene glycol and 50% water. To facilitate recovery from the anaesthetic and permit acclimation to the chamber the $T_b$ of the animal was maintained at 35-37°C for 30 minutes before the experiment began.

**Figure 2.1.** Apparatus for *in vivo* experiments including the water-jacketed temperature control tube, pneumotachograph (P), differential pressure transducer (T), the impedance/ EKG metre (I), and the thermocouple transducer ($T_b$). This diagram shows the head-out body plethysmograph method of measuring respiratory activity.

Due to the large range in body size and level of activity in individuals of different species and ages, three different techniques were used to monitor breathing. For the first, head-out body plethysmography, the animal was secured with the body inside and the head
outside the water-jacketed temperature control cylinder (Mortola, 1984). The end of the water-jacketed cylinder was covered with a flexible latex collar, sealed with an o-ring, through which the head of animal protruded (Figure 2.1). The protruding head of the animal was then encased in a second water-jacketed cylinder, open to the air, that maintained the head and body at the same temperature. The pressure difference between the inside of the water-jacketed cylinder and the outside room air, created by the breathing movements of the animal, was detected by the pneumotachograph attached to a Validyne differential pressure transducer. Calibration of the differential pressure detected by the pneumotachograph was achieved by injecting known volumes of air into the cylinder and measuring the voltage change that resulted (Mortola, 1984).

For the second technique, whole body plethysmography, the animal was placed completely inside the temperature control cylinder and the front opening sealed with a rubber stopper. The electrode and thermocouple wires were passed through a port in the top of the cylinder which permitted the animal to move freely inside the cylinder. Air was passed through the chamber at a constant rate that insured adequate ventilation of the space occupied by the animal without obscuring the changes in pressure associated with inspiration and expiration. Inflowing air was rendered the same temperature as the water surrounding the animal by passing through a length of copper tubing placed in the Lauda refrigerated circulator. Similar to head-out body plethysmography, breathing was monitored by measuring the pressure difference between the inside and the outside of the cylinder using a pneumotachograph attached to a Validyne transducer. However, accurate volume calibration of the signal was impossible using this technique because of the fluctuations in pressure inside the cylinder caused by the non-respiratory movements of the animal. The third method of obtaining breathing traces measured the impedance (resistance) across the chest wall of the animal using the electrocardiogram electrodes. In this technique a small amount of alternating current, too small to be detected by the animal, was passed through the chest wall. The resistance to the current increased during inspiration, as the lungs are filled, and decreased during expiration, as the lungs were emptied. This change in resistance was measured with an impedance converter (UFI, Model 2991). Using head-out body plethysmography both frequency of ventilation and the tidal volume of each breath could be calculated. Using whole body plethysmography and impedance measurements, only the frequency of
ventilation could be determined since accurate volume calibration was exceedingly difficult with these two methods.

The breathing and ECG signals were amplified (1,000-10,000X) and filtered. Body temperature was transduced using the Physitemp analog converter. All signals were recorded using DataQ Instruments WindaqPro data acquisition software (Version 2.71) at a sampling rate of 500 samples/second/channel.

2.3.3. Protocol
Once instrumented and recovered the animal was cooled at a rate of 0.2-0.4°C/minute until ventilation ceased (Table 2.1). Respiratory arrest was defined as 10 minutes with no breathing activity. The $T_B$ of the animal was kept 0-2°C below the temperature at which arrest occurred for 10 minutes. The animal was then progressively re-warmed at a rate of ~0.4°C/minute until $T_B$ reached 35°C.

2.3.4. Analysis
Raw signals were integrated using DataQ Instruments Windaq Waveform Browser (Version 2.19) and Advanced Codas Calculation Package (Version 3.25). At least 20 breaths were analysed for each degree ($T_B$) at higher temperatures during the cooling and re-warming protocol. At very low $T_B$, breathing frequency ($f_R$) was low, in which case the whole trace for each degree was analysed. Breathing frequency and heart rate ($f_H$) were determined for each degree $T_B$ during cooling and re-warming and the mean for each age group of each species was calculated. The mean $T_B$ at which respiratory arrest occurred, called "minimum $T_B"", and also the rates of survival were determined for each age group of each species. Tidal volume ($V_T$) and inspiratory time ($T_i$) were determined for a) last breath before respiratory arrest b) first breath after respiratory arrest (upon re-warming) c) 5 breaths at the same $T_B$ during cooling as the first breath upon re-warming and d) 5 breaths at 35°C (Figure 2.2).

2.3.5. Statistics
Rates of cooling to the minimum $T_B$ were compared using a one-way ANOVA followed by a pair-wise multiple comparisons procedure (Tukey test). This test was also used to
determine differences in body mass between the different age groups of each species. A one-way repeated measures ANOVA followed by a Bonferroni post hoc test was used to detect differences in \( f_R \) and \( f_H \) during cooling and re-warming. Values were compared to control values taken at 35°C before cooling began. Data that was not normally distributed was analysed using a non-parametric repeated measures rank ANOVA followed by Dunn's post hoc test. Starting \( f_R \) (at 35°C), \( f_H \) at the minimum \( T_B \) and the \( T_B \) of the last breath during cooling and the first breath upon re-warming were compared within each species (between age groups) using a one-way ANOVA followed by a pairwise Tukey post hoc test. Tidal volume (\( V_T \)) and inspiratory time (\( T_I \)) of breaths at various points during cooling and re-warming were compared using a one-way repeated measures ANOVA followed by a Bonferroni post hoc test.

**Figure 2.2.** Schematic of how respiratory variables were determined. Flow of the breath (inspiration upwards) is indicated by the raw trace at the top and the integrated trace representing volume of the breath is indicated on the bottom. Zero flow is indicated by dashed line.
2.4. RESULTS

2.4.1. Cooling to the minimum $T_b$
Juvenile rats and hamsters were cooled to a temperature at which breathing arrested in 70-165 minutes. Average rates of cooling for rats and hamsters ranged from 0.21± 0.03 to 0.37± 0.03°C/ min, however there was no significant effect of age or species on the rate of cooling except that P14 rats cooled significantly more quickly than P18 rats, P21-23 hamsters and P26-28 hamsters (Table 2.1). P14 rats had significantly lower body mass (28.0± 1.5 g) than P16 and P18-20 rats (34.3± 1.3 g and 38.1± 1.9 g respectively). Hamsters, however, were not of significantly different mass at P15-20 and P21-23 (16.9± 3.1 g and 23.5± 5.8 g) while P26-28 hamsters were significantly larger (45.9± 2.6 g). The rate of cooling for each age group was nearly constant over the $T_b$ range until $T_b$ plateaued near the minimum temperature (Figure 2.3). This plateau was likely a result of the reduction in the thermal gradient caused by the minimum temperature of the water flowing through the cylinder which was ~2°C. For the first 15°C of cooling all animals, with the exception of P14 rats, exhibited rigorous shivering in response to the cold. Animals also adopted postures within the cylinder that appeared to minimise their surface area while maximising their distance from the cylinder walls.

2.4.2. Breathing frequency and heart rate during cooling
Juvenile rats in all age groups breathed at similar frequencies at 35°C, though with considerable individual variability (120.6± 18.7 breaths/ min; Figure 2.4). There were, however, significant differences in breathing frequency ($f_R$) at 35°C between hamsters of different ages (Table 2.1; Figure 2.5). The $f_R$ of P26-28 hamsters was 205.7± 22.0 breaths/ min while that of P15-20 and P21-23 hamsters were 109.3± 28.2 and 68.3± 12.6 breaths/ min respectively. There were no significant differences in the $f_H$ of rats and hamsters of different age groups at 35°C. There was, however, a general trend towards higher $f_H$ in older animals of both species (Table 2.1; Figure 2.6; Figure 2.7).

During initial cooling, between $T_b$ of 35°C and 20-25°C, $f_R$ remained constant or was slightly elevated in all age groups and species (Figure 2.4; Figure 2.5). This effect was most noticeable in P26-28 hamsters for which $f_R$ was not significantly lower than
euthermic levels until $T_B$ had declined to 15°C (Figure 2.5). Below ~20-25°C, $f_R$ decreased in a roughly linear fashion in all age groups of both species until respiratory arrest occurred (Figure 2.4; Figure 2.5). In rats, $f_H$ remained constant or was slightly elevated for the first 5-10°C of cooling in all age groups and then declined in a linear fashion to a mean of 8.18± 2.35 beats/min at the minimum $T_B$ (Figure 2.6). In hamsters $f_H$ declined within the first few degrees of cooling rather than remaining constant as in rats. Heart rate in P26-28 hamsters had decreased significantly from euthermic levels by a $T_B$ of 33°C (Figure 2.7). The $f_H$ of hamsters in all age groups declined in a linear fashion with $T_B$ until a mean of 10.0± 1.35 beats/min at the minimum $T_B$.

2.4.3. Respiratory arrest and autoresuscitation
Species but not age had an effect on the $T_B$ at which animals took their last breath (Figure 2.8). Since age was not a determining factor for this variable the data for all ages was pooled for each species. Rats took their last breath at a mean $T_B$ of 9.1± 0.94°C while hamsters breathed to a significantly lower mean $T_B$ of 3.8± 0.31°C.

Of P14 rats, 75% autoresuscitated from hypothermia-induced respiratory arrest whereas 25% of P16, and none of P18-20 rats, autoresuscitated. All P15-20 hamsters tested autoresuscitated whereas only 20% of P21-23 hamsters autoresuscitated (Figure 2.9). All P26-28 hamsters recovered completely from hypothermia. If, however, P26-28 hamsters were left at the minimum $T_B$ for 30 minutes of no breathing instead of the usual 10 minutes they failed to recover (n=3).

Species but not age had an effect on the $T_B$ at which animals took their first breath after re-warming from the minimum $T_B$ (Figure 2.8). Rats took their first breath during re-warming at a mean $T_B$ of 13.7± 1.1°C while hamsters took their first breath at a significantly lower $T_B$ of 10.2± 0.47°C. The $T_B$ at which autoresuscitation occurred was found to be significantly different from the $T_B$ at which respiratory arrest occurred in hamsters of all age groups but not in rats.

2.4.4. Last breath before respiratory arrest and first breath after respiratory arrest
In both species tested the last breath during cooling and the first breath after re-
warming from the minimum \( T_B \) were of higher tidal volume (\( V_T \)) and longer inspiratory time (\( T_I \)) than breaths at 35°C (Figure 2.2; Figure 2.10). Due to considerable variability in the actual values of \( V_T \), this variable is presented as % of \( V_T \) at 35°C (before cooling).

In both rats and hamsters, \( T_I \) of the last breath was significantly longer (1.2± 0.15 and 1.8± 0.10 sec, respectively) than mean \( T_I \) at 35°C (0.26± 0.06 and 0.12 sec, respectively). The \( V_T \) of the last breath was larger than mean \( V_T \) at 35°C in both rats and hamsters (247.8± 63.7 and 302.3± 133 % of control, respectively) however in neither species was this difference significant (Figure 2.10).

In rats, \( T_I \) of the first breath during re-warming from the minimum \( T_B \) was significantly longer (1.1± 0.34 sec) than mean \( T_I \) at 35°C (0.26± 0.06 sec) but not significantly longer than the mean \( T_I \) at that same \( T_B \) during cooling (0.57± 0.04). Whereas in hamsters \( T_I \) of the first breath after was significantly longer (1.5± 0.17 sec) than both the mean \( T_I \) at 35°C (0.12± 0.0002 sec) and also the mean \( T_I \) at that same \( T_B \) during cooling (0.46± 0.05 sec). \( V_T \) of the first breath was significantly larger than \( V_T \) at 35°C in both rats and hamsters (399.3± 46.4 and 578.5± 172.5% of control, respectively) but not significantly higher than mean \( V_T \) at that \( T_B \) during cooling (252± 89.1 and 214.3± 26.2 % of control, respectively; Figure 2.10).

2.4.5. Re-warming from minimum \( T_B \)

Re-warming from the minimum \( T_B \) was accomplished in 62-85 minutes at a rate ranging from 0.37± 0.03 to 0.46°C/ minute (Table 2.1). Rats and hamsters of all age groups re-warmed at the same rate and reached \( T_B \) of 35°C after approximately the same length of time despite the difference in the \( T_B \) from which they were re-warmed (minimum \( T_B \) for hamsters was significantly lower than for rats; Figure 2.3).

As discussed above, not all rats and hamsters autoresuscitated from hypothermia-induced respiratory arrest (Figure 2.9). Those individuals that were destined to die failed to take even a single breath upon re-warming (Figure 2.4; Figure 2.5). However, while \( f_R \) remained at zero for those animals that failed to autoresuscitate, \( f_H \) increased throughout the initial stages of re-warming as it did in those animals that ultimately
recovered (Figure 2.6; Figure 2.7). At the mean $T_b$ of first breath upon re-warming for each species there was no significant difference between the mean $f_h$ of those animals that were destined to fail to autoresuscitate and those destined to autoresuscitate and ultimately survive.

For those individuals that autoresuscitated from hypothermia-induced respiratory arrest, $f_R$ and $f_H$ increased in a similar fashion during re-warming as they decreased during cooling. Once breathing re-started, the frequency increased in a near-linear fashion until it reached euthermic levels. Normal breathing frequencies were observed in P26-28 hamsters at $T_b < 25^\circ C$. Mean breathing frequencies at $T_b > 24^\circ C$ were not significantly different from the $f_R$ at $35^\circ C$ (Figure 2.5). In both rats and hamsters the $f_R$ at $35^\circ C$ after re-warming appeared to be the same as $f_R$ at $35^\circ C$ before cooling (Figure 2.4; Figure 2.5). During initial re-warming, $f_H$ increased with $T_b$ before breathing re-started however this increase in $f_H$ prior to autoresuscitation of breathing was only significant in P16 rats and P15-20 hamsters. Heart rate increased in a linear fashion as $T_b$ increased to $35^\circ C$. Heart rate at $35^\circ C$ at the end of the experiment, in all cases, was similar to $f_H$ before cooling began (Figure 2.6; Figure 2.7).

Both $f_R$ and $f_H$ showed a 5-10$^\circ C$ hysteresis when data from cooling was compared to that of re-warming. Thus, during re-warming any given $f_R$ or $f_H$ occurred at a $T_b$ 5-10$^\circ C$ higher than the $T_b$ at which that same $f_R$ or $f_H$ occurred during cooling (Figure 2.4; Figure 2.5; Figure 2.6; Figure 2.7). As a result of the hysteresis animals autoresuscitated on average 5$^\circ C$ $T_b$ warmer than the $T_b$ at which they underwent respiratory arrest.
Table 2.1. Cardiorespiratory variables for P14, P16 and P18-20 rats and P15-20, P21-23 and P26-28 hamsters. Results are presented as mean values ± SEM. Sample size is in brackets.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Rat</th>
<th>Hamster</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass (g)</td>
<td>28.0±1.5 (4)</td>
<td>34.3±1.3 (4)</td>
</tr>
<tr>
<td>Rate of cooling (°C/ min)</td>
<td>0.37±0.03 (4)</td>
<td>0.29±0.04 (4)</td>
</tr>
<tr>
<td>Time to reach minimum TB (min)</td>
<td>71.3±9.7 (4)</td>
<td>91.3±12.6 (4)</td>
</tr>
<tr>
<td>Rate of re-warming (°C/ min)</td>
<td>0.44±0.036 (3)</td>
<td>0.41 (1)</td>
</tr>
<tr>
<td>Time to reach 35°C (min)</td>
<td>62.3±5.6 (3)</td>
<td>70.0 (1)</td>
</tr>
<tr>
<td>TB at which breathing arrested (°C)</td>
<td>9.4±2.0 (4)</td>
<td>9.9±1.5 (4)</td>
</tr>
<tr>
<td>TB at which breathing re-started (°C)</td>
<td>13.5±2.8 (3)</td>
<td>16.8 (1)</td>
</tr>
<tr>
<td>fR at 35°C (breaths/ min)</td>
<td>102.9±13.3 (4)</td>
<td>160.7±91.9 (4)</td>
</tr>
<tr>
<td>fH at 35°C (beats/ min)</td>
<td>311.4±29.1 (4)</td>
<td>373.3±21.0 (4)</td>
</tr>
<tr>
<td>fH at minimum TB (beats/ min)</td>
<td>8.4±2.0 (4)</td>
<td>3.4±1.1 (4)</td>
</tr>
<tr>
<td>Survival (% autoresuscitated)</td>
<td>75</td>
<td>25</td>
</tr>
</tbody>
</table>

a = significantly different from both P15-20 and P21-23 hamsters
b = significantly different from P18 rats, P21-23 hamsters and P26-28 hamster
c = significantly different from P16 and P18 rats
d = significantly different from P15-20 and P21-23 hamsters
Figure 2.3. Time course of change in mean $T_b$ during progressive hypothermia and subsequent re-warming to 35°C for A) rats aged 14, 16, 18-20 days and B) hamsters aged 15-20, 21-23 and 26-28 days. All data are presented as mean ± SEM.
Figure 2.4. Breathing frequency during cooling and re-warming in A) P14 B) P16 C) P18-20 rats. All data are presented as mean ± SEM.
Figure 2.5. Breathing frequency during cooling and re-warming in A) P15-20 B) P21-23 C) P26-28 hamsters. All data are presented as mean ± SEM. * denotes a significant difference from 35°C (P<0.05)
Figure 2.6. Heart rate during cooling and re-warming in A) P14 B) P16 C) P18-20 rats. All data are presented as mean ± SEM.
Figure 2.7. Heart rate during cooling and re-warming in A) P15-20 B) P21-23 C) P26-28 hamsters. All data are presented as mean ± SEM. * denotes a significant difference from 35°C (P≤0.05)
Figure 2.8. $T_b$ of last breath before respiratory arrest and first breath after respiratory arrest (autoresuscitation) for A) rats aged 14, 16, 18-20 days and B) hamsters aged 15-20, 21-23 and 26-28 days. All data are presented as mean ± SEM. * denotes a significant difference from the $T_b$ of last breath before respiratory arrest ($P<0.05$)
Figure 2.9. The relationship between age and % survival of A) rats aged 14, 16, 18-20 days and B) hamsters aged 15-20, 21-23 and 26-28 days.
Table 2.2. Tidal volume ($V_T$) and inspiratory time ($T_i$) of the last breath during cooling, the first breath upon re-warming and a breath at the same $T_b$ during cooling as the first breath upon re-warming. Results are normalised to % of values at 35°C and presented as mean ± SEM. * denotes a significant difference from control (at 35°C) † denotes a significant difference from breaths at same $T_b$ during cooling as first breath upon re-warming (P≤0.05)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Species</th>
<th>Breath at 35°C (control)</th>
<th>Last breath</th>
<th>First breath</th>
<th>Breath at same $T_b$ during cooling as first breath upon re-warming</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tidal volume (% of control)</td>
<td>rat</td>
<td>100</td>
<td>248±64</td>
<td>399±47*</td>
<td>252±89</td>
</tr>
<tr>
<td></td>
<td>hamster</td>
<td>100</td>
<td>302±133</td>
<td>579±173*</td>
<td>214±26</td>
</tr>
<tr>
<td>Inspiratory time (% of control)</td>
<td>rat</td>
<td>100</td>
<td>503±103*</td>
<td>451±63*</td>
<td>248±53</td>
</tr>
<tr>
<td></td>
<td>hamster</td>
<td>100</td>
<td>1481±84†</td>
<td>1188±133*</td>
<td>376±42*</td>
</tr>
</tbody>
</table>
Figure 2.10. Tidal volume ($V_T$) and inspiratory time ($T_I$) of the last breath during cooling, the first breath upon re-warming and a breath at the same $T_B$ during cooling as the first breath upon re-warming for A) rats aged 14-20 days and B) hamsters aged 21-28 days. $V_T$ is normalised as % of $V_T$ at $35^\circ$C. Air flow rate for each inspiration is indicated by the slope of the line. Results are presented as mean values± SEM. * denotes a significant difference from breaths at $35^\circ$C. † denotes a significant difference from breaths at same $T_B$ during cooling as first breath upon re-warming ($P \leq 0.05$)
2.5. DISCUSSION

Rats and hamsters give birth to altricial young that are completely dependent on their mother for food, warmth and protection. In just 3 weeks, neonatal rats and hamsters develop into independent organisms, capable of effective thermoregulation, feeding themselves and moving about their environment. Neonatal and adult mammals are faced with very different challenges and therefore the characteristics that promote survival are different at each developmental stage. In general, neonates tend to tolerate adverse situations, such as reduced food availability or cold temperatures, whereas adult mammals avoid them. Among the developmental changes that occur in the first few weeks of life are the development of endothermy and homeothermy, the loss of neonatal cold tolerance, and the loss of the ability to re-start breathing in hypothermia, a process called autoresuscitation.

2.5.1. Developmental differences between rats and hamsters

The average growth rates of rats and hamsters between birth and weaning are ~1.5 g/day and ~1.2 g/day, respectively. Rats are considerably larger than hamsters at birth (5.0-6.0 g in contrast to 2.5-3.0 g) and as adults (250-350 g in contrast to 100-140 g). Rats are born after 21 days of gestation whereas hamsters are born after only 16 days of gestation, so particularly during the early neonatal period hamsters may be less developed than rats. Neonatal rats have some capacity to thermoregulate using brown adipose tissue (BAT), or "non-shivering" thermogenesis by 2 days of age while hamsters depend exclusively upon behavioural mechanisms of temperature regulation (such as huddling), as they have no BAT until 10-12 days of age (Spiers & Adair, 1986; Blumberg, 1997; Sokoloff et al., 2000). Shivering thermogenesis occurs first at 12 days of age in both rats and hamsters but thermoregulation is inadequate to maintain Tb in room temperature air until they are fully furred at 15-18 days of age (Fairfield, 1948; Spiers & Adair, 1986; Sokoloff et al., 2000). The development of endothermy and adult-type homeothermy in juvenile rats and hamsters is accompanied by an increase in the normal body temperature of these animals. Newborn rats and hamsters normally have a Tb of 33-35°C, approximately 2.0-5.0°C lower than that of adults (Mortola, 2001; Rogalska & Caputa, 2004). Juveniles of both species are typically weaned at 21 days of age and are sexually mature at between 30 and 50 days of age (Baker et al., 1979; Van...
Hoosier & McPherson, 1979). By about 15 days of age rats and hamsters are 13-15% of their adult mass and appear to be at similar stages of development.

2.5.2. **Effect of age and species on cardiorespiratory variables at 35°C**
The $f_R$ and $f_H$ at euthermic temperatures were much higher in older animals than in younger animals of both species (Table 2.1; Figure 2.6; Figure 2.7). Similar observations were made by Adolph (1951) who reported gradual increases in $f_R$ and $f_H$ over development in rats and hamsters between 14 days of age to 30 days of age, a similar age range to those in the current study. Metabolic rate and ventilation reportedly increase in the first 3 weeks of life in rats and hamsters before gradually declining to adult levels (Mortola, 1984; Mortola, 2001). Although neither metabolic rate nor ventilation were measured in the current study, our observations of increasing $f_R$ and $f_H$ are consistent with an increase in metabolic rate between the ages of 14 and 20 days in rats and 15 and 28 days in hamsters. The reported increase in metabolic rate over development in these animals may reflect the development of endothermy and the maintenance of a higher euthermic $T_B$, similar to that of adult mammals.

2.5.3. **Effect of progressive hypothermia on cardiorespiratory variables**
When the $T_B$ of ectothermic animals declines, metabolic rate declines in proportion to $T_B$. Endothermic animals such as mammals, however, usually respond to a decline in $T_B$ with an initial increase in metabolic rate as they attempt to return $T_B$ to normal using shivering and non-shivering thermogenesis (Fairfield, 1948; Adolph & Lawrow, 1951; Spiers & Adair, 1986; Blumberg et al., 1997; Mortola & Frappell, 2000). If the decline in $T_B$ continues despite thermogenesis, eventually the effects of low temperature on metabolism exceed the thermogenic abilities of the animal and metabolic rate begins to decline in proportion to $T_B$ (Adolph, 1961; Mortola & Frappell, 2000). However, if mammals are prevented from using shivering and/or non-shivering thermogenesis (eg. in anaesthesia) metabolic rate declines in proportion to $T_B$ as it does in ectotherms (Kiley et al., 1984; Osborne & Milsom, 1993; Blumberg et al., 1997). Similarly, very young mammals not yet capable of thermogenesis, behave like anaesthetised adult mammals and ectotherms and metabolic rate declines in proportion with $T_B$, even during the initial stages of cooling (Fairfield, 1948; Spiers & Adair, 1986; Blumberg, 1997).
During the initial stages of cooling of juvenile rats and hamsters in the current study, $f_R$ was observed to remain at euthermic levels until $T_B$ fell to 16-25°C rather than declining linearly with $T_B$. While this was observed in juveniles of all ages and both species, older rats and hamsters typically maintained high $f_R$ until they reached lower $T_B$ than younger rats and hamsters (Figure 2.4; Figure 2.5). Similar observations were reported for rats between 2 days of age and adulthood and hamsters between 9 days of age and adulthood, however, in hamsters younger than 9 days, $f_R$ reportedly declined in proportion to $T_B$, even during initial cooling (Adolph, 1951; Adolph & Lawrow, 1951; Adolph, 1961; Tattersall & Milsom, 2003). Changes in $f_R$ are usually matched to changes in $V_E$ in hypothermia because $V_T$ remains constant or is slightly elevated (Osborne & Milsom, 1993; Corcoran & Milsom, 2003; Tattersall & Milsom, 2003). Similarly, $V_E$ is typically matched to oxygen consumption ($V_O_2$) in hypothermia, both during the initial thermogenic period, when metabolic rate is elevated, and after thermogenesis fails when metabolic rate begins to decline (Adolph & Lawrow, 1951; Mortola & Frappell, 2000). This strongly suggests that in the current study, $f_R$ was maintained at normal or elevated levels during the first 10-20°C of cooling in an effort to meet the high metabolic requirements of thermogenesis. Breathing frequency was likely higher, and remained elevated for longer during cooling, in older animals of both species because these individuals had an increased capacity for thermogenesis in comparison to younger animals. Below $T_B$ of 16-25°C, $f_R$ was observed to decline with $T_B$ in a linear fashion suggesting that, at these low temperatures, thermogenesis failed and metabolic rate began to decline. Such declines in the frequency of breathing and therefore $V_E$ are usually matched to metabolic rate, even at these very low $T_B$, with the result that the metabolic needs of the animal are met until respiratory arrest occurs (Rosenhain & Penrod, 1951; Mortola & Frappell, 2000).

Strangely, while $f_H$ was maintained at euthermic levels for the first 5°C of cooling in rats of each age group in this study, and also in rats aged 0-6 days (Tattersall & Milsom, 2003), $f_H$ of hamsters of all age groups in the current study declined in proportion to $T_B$ throughout the experiment (Figure 2.7). This was in contrast to what was observed in $f_R$ in juvenile hamsters, which was maintained at euthermic levels, or elevated above euthermic levels, for the first 10-20°C of cooling. Similarly, Adolph (1951) found that,
while \( fr \) was maintained at euthermic levels in hamsters 9-30 days of age until \( TB \) fell to 25°C, \( fH \) declined with \( TB \) starting at 35°C. In adult hamsters, however, \( fr \) and \( fH \) were matched throughout cooling which is consistent with the maintenance of elevated metabolic rate for thermogenesis (Adolph & Lawrow, 1951). This implies that for some reason, changes in \( fH \) and metabolic rate were uncoupled in hamsters younger than 30 days of age. Alternatively, this could indicate that the elevated \( fr \) and \( VE \), observed in juvenile hamsters during the first 10-20°C of cooling, were not matched to an increase in metabolic rate but may have been an attempt to maintain pH at normal levels despite the decline in \( TB \) (White, 1981; Mortola & Frappell, 2000; Tattersall & Milsom, 2003).

Below \( TB \) of ~30°C, \( fH \) began to decline in proportion with temperature in rats of all age groups. As discussed above, \( fH \) declined from the start of the experiment (35°C) in hamsters of all age groups. Once thermogenesis failed during progressive hypothermia, \( fr \) and \( fH \) declined with \( TB \) in proportion to one another. (Adolph, 1948b, 1951; Adolph & Lawrow, 1951; Adolph, 1961; Corcoran & Milsom, 2003; Tattersall & Milsom, 2003).

2.5.4. Respiratory arrest in hypothermia

There is considerable evidence to indicate that respiratory arrest in hypothermia occurs as a result of failure of the CRG for breathing located in the brainstems of mammals (Mellen et al., 2002; Milsom et al., 2002). Neonatal and juvenile rats underwent respiratory arrest at \( TB \) of ~10 whereas adult rats reportedly failed to breathe at \( TB \) of 13-16 (Adolph, 1948a; Fairfield, 1948; Tattersall & Milsom, 2003). This implies that the CRG for breathing in juvenile rats 14-20 days old was more tolerant of low \( TB \) than the CRG of adult rats but of similar tolerance to that of P0-6 rats. In contrast, juvenile hamsters underwent respiratory arrest at a \( TB \) of 3.8± 0.31°C, very similar to that reported for both neonatal and adult hamsters (Adolph & Lawrow, 1951; Kristofferersson & Soivio, 1966; Corcoran & Milsom, 2003). Therefore it appears that, the CRG of juvenile and adult hamsters retained neonatal cold tolerance rather than losing it over development.

2.5.5. Respiratory arrest in hypothermia is not accompanied by cardiac arrest

After respiratory arrest at the minimum \( TB \), the heart continued to beat at a very low frequency in all juvenile rats and hamsters in the current study. Heart rate at these very low temperatures varied greatly between individuals but there was no significant
difference between \( f_H \) of those individuals that were destined to autoresuscitate upon re-warming, and those that were destined to fail to autoresuscitate, either at the \( T_B \) of arrest or at the \( T_B \) of autoresuscitation. Similarly, the heart reportedly continued to beat at low frequencies after respiratory arrest had occurred in neonatal rats and hamsters, as well as in adult dogs (Rosenhain & Penrod, 1951; Corcoran & Milsom, 2003; Tattersall & Milsom, 2003). However, Adolph (1948a) and Fairfield (1948) reported that the hearts of neonatal rats and hamsters (various ages between 0 and 30 days of age) stopped beating at \( T_B \) just below that at which they reported breathing movements ceased and/or \( V_O_2 \) reached zero. Heart rate and the presence or absence of arrhythmia were not considered by these authors to be correlated with the survival of the animal after re-warming (Adolph, 1948b; Fairfield, 1948; Adolph, 1951). A recent paper disputed these findings, demonstrating that during apnoea in deep hypothermia (\( T_B = 2\sim 3^\circ C \)) the hearts of mammalian neonates (\( P_0-10 \)) continued to beat in the absence of breathing and that the continuation of heart beats was required for the survival of these animals (Hill, 2000). It may be that Adolph (1948a; 1951) and Fairfield (1948) were unable to see heart beats at these very low \( T_B \) because the amplitude of the ECG signal was greatly reduced, and the shape of the signal greatly distorted, when compared to the ECG at euthermic \( T_B \).

2.5.6. Autoresuscitation from hypothermia-induced respiratory arrest
When neonatal mammals are re-warmed from hypothermia-induced respiratory arrest they re-start breathing and, upon re-warming to normal \( T_B \), recover completely (Corcoran & Milsom, 2003; Tattersall & Milsom, 2003). In the current study the ability to autoresuscitate was lost in rats between P14 and P20 (Figure 2.9). In hamsters the window was much later in development than in rats. According to Adolph (1951) hamsters lost the ability to autoresuscitate between P30 and adulthood. However, between 21 and 23 days of age very few hamsters in the current study autoresuscitated indicating that, while in general hamsters were capable of autoresuscitation until later in life than rats, these animals underwent a period of sensitivity to hypothermia between P21 and P23. It may be that the CRG for breathing was under stronger inhibitory drive at this age resulting in a "critical period" during which the CRG was unable to re-start if respiratory failure occurred (Liu & Wong-Riley, 2002; Liu & Wong-Riley, 2003).
Autoresuscitation from hypothermia-induced respiratory arrest occurred in rats at higher body temperatures than in hamsters and in all age groups of both species, approximately 5°C higher than the T₅ at which breathing arrested during cooling (Figure 2.8). Thermal hysteresis was also observed in the breathing frequency and heart rate of rats and hamsters of all age groups in the current study, and in numerous previous studies (Adolph, 1951; Corcoran & Milsom, 2003; Tattersall & Milsom, 2003). Hysteresis resulted in any given value of breathing frequency or heart rate occurring during rewarming at a temperature 5-10°C higher than the T₅ at which that value occurred during cooling. This degree of thermal hysteresis would have had interesting implications if it represented an actual difference in the relationships between cardiorespiratory variables and temperature during cooling and re-warming, however, it is likely that the hysteresis observed was not true hysteresis. Instead, the observed difference in cardiorespiratory variables during cooling and re-warming was likely a result of the discrepancy between the rectal temperature that was measured and the brain or heart temperature. Body temperature measured in different locations on the body (e.g. intraperitoneal, intrascapular and rectal) has been found to vary by up to 10°C during changes in ambient temperature (Spiers & Adair, 1986; Blumberg & Sokoloff, 1998). In the current study, the brain and heart temperatures likely lagged behind the measured rectal temperature during cooling and re-warming with the result that the rectal temperature was not directly related to the brain and heart temperatures during the initial stages of both cooling and re-warming. Since cardiorespiratory variables such as breathing frequency are likely dependent upon brain temperature, it is likely that these variables are not directly related to rectal T₅ during initial cooling and re-warming. This could explain both the sigmoidal shape of the curves shown in Figures 2.4, 2.5, 2.6 and 2.7 and also the apparent thermal hysteresis exhibited in both the breathing frequency and heart rate of rats and hamsters. Further evidence in support of the hypothesis that the apparent hysteresis observed in the current study was a result of the discrepancy between rectal and brain/heart T₅ can be found in in vitro studies, in which brain temperature can be determined exactly. Little or no thermal hysteresis has been observed in fictive breathing frequency during cooling and re-warming in in vitro studies using either the sagittal slice preparation or the en bloc preparation (Mellen et al., 2002; Chapter 3).
According to Adolph (1948a; 1951), as long as the duration of respiratory arrest did not exceed 2-3 hours, the actual time spent without breathing had no effect on the rate of survival of neonatal rats and hamsters. These observations are consistent with those of Hill (2000) who reported that neonatal mammals exchange gases passively through the open glottis after visible respiration has ceased in hypothermia and therefore can remain at low $T_B$, without breathing, indefinitely. Respiratory arrest was arbitrarily defined in the current study as no breathing activity for 10 minutes at the minimum temperature, after which the animal was re-warmed. Interestingly, if juvenile hamsters (P26-28) were held at the minimum temperature for 30 minutes instead of 10 minutes the survival rate fell from 100% to zero. Thus the duration of respiratory arrest was of essential importance to survival in these older animals implying that other factors besides the ability of the CRG to re-start were involved in their survival.

2.5.7. Effect of re-warming on cardiorespiratory variables
During the initial stages of re-warming from the minimum $T_B$, the $f_H$ of all juvenile rats and hamsters increased with temperature. For those animals that re-started breathing during the re-warming process, $f_H$ continued to increase with $T_B$ in a near-linear fashion until the animal reached 35°C (Figure 2.6; Figure 2.7). However, in those animals that failed to autoresuscitate during re-warming, the heart continued to beat at a low rate until ~25°C when $f_H$ began to decline. This indicates that the critical difference between those rats and hamsters that recovered completely from hypothermia and those that failed to recover was not a difference in heart function.

In those animals that autoresuscitated, $f_R$ increased with $T_B$ in a near-linear fashion for the first 10-15°C of re-warming. At $T_B$ ~25°C, $f_R$ had reached euthermic levels and then remained constant or slightly elevated until $T_B$ reached 35°C. This implies that these animals were attempting to increase $T_B$ using thermogenesis, requiring higher $V_O_2$ and therefore higher $f_R$ to meet their metabolic needs. In hamsters, however, elevated $f_R$ was not accompanied with elevated $f_H$ indicating that, in these animals, $f_R$ may not have been matched to metabolic rate. Instead, $f_R$ in hamsters may have been elevated in an attempt to regulate pH during these abrupt changes in $T_B$ (White, 1981; Mortola & Frappell, 2000; Tattersall & Milsom, 2003).
2.5.8. Cold tolerance, thermoregulation and the ability to autoresuscitate

The work of Adolph and others (1948a; 1948b; 1951; 1961) comparing the cold tolerance of neonatal and adult mammals has led to the conclusion that neonatal mammals are more tolerant of low \( T_B \) than adults and that neonatal cold tolerance is gradually lost over development. Adolph (1951) observed that the age at which the lethal \( T_B \) of juveniles reached that of the adult was a good predictor of the age at which the ability to re-start breathing was lost (ie. ~19 days of age in rats and ~30 days of age in hamsters). However, in the current study, no difference in the \( T_B \) at which animals of different ages underwent respiratory arrest was observed. Hamsters aged 15-28 days stopped breathing at a \( T_B \) of ~3.5°C whereas rats aged 14-20 days stopped breathing around 10°C. While hamsters of all age groups were more tolerant of low \( T_B \) than rats of all age groups there was no developmental difference in either species implying that the CRG of neither species lost the ability to tolerate low \( T_B \), at least in the age groups studied. Thus the ability to autoresuscitate in rats and hamsters appeared to be uncoupled from the ability to tolerate low \( T_B \) without undergoing respiratory arrest.

The loss of the ability to autoresuscitate in rats and hamsters, while it was not accompanied by a decline in cold tolerance in the current study, did appear to occur as juveniles developed adult type homeothermy. Prior to 15-17 days of age neither rats nor hamsters are capable of maintaining \( T_B \) in room temperature air (Fairfield, 1948; Spiers & Adair, 1986; Sokoloff et al., 2000). By ~20 days and ~28 days of age rats and hamsters are able to thermoregulate about as well as adults, despite being much smaller (~30% of their adult weight). Thus the ability to autoresuscitate is lost in these animals at about the same point in development as they gain the ability to thermoregulate and therefore avoid low \( T_B \).

2.5.9. Is hypoxia a factor in hypothermia?

In addition to cold tolerance, many authors have observed that neonatal mammals, especially those that are altricial, are extraordinarily tolerant of hypoxia and even anoxia (Adolph, 1948b; Nioka & Chance, 1991; Fewell et al., 2000; Mortola, 2001). As such hypoxia tolerance has long been implicated as a contributing factor to the ability of mammalian neonates to tolerate very low \( T_B \) and hypothermic respiratory arrest. There is, however, considerable evidence to indicate that neonatal mammals do not suffer
from hypoxia during hypothermia-induced respiratory arrest. Hill (2000) reported that neonatal mammals exchanged O₂ and CO₂ through their open glottis during hypothermia induced apnoea and that they appeared to have functional circulatory systems adequate to meet the reduced metabolic needs of the animal at low Tb. Therefore, at least in neonatal mammals, hypoxia tolerance does not appear to play a role in survival of hypothermia-induced respiratory arrest. The same may also be true in adult mammals since some authors have reported that both blood and tissue PO₂ remain normal when adult mammals were subjected to severe hypothermia, indicating that ventilation was adequate to meet their oxygen requirements (Rosenhain & Penrod, 1951; Byon & Adolph, 1961).

2.5.10. Does gasping occur in hypothermia and recovery from respiratory arrest? Gasping is a type of breathing observed when mammals are exposed to prolonged anoxia or asphyxia and appears to be the final effort of the respiratory system to recover from apnoeic episodes, such as those that occur in sudden infant death syndrome (Thach et al., 1991). Gasps are characterised by their short inspiratory duration (Ti) and their large inspiratory volume (VT) in comparison to eupnoeic breaths (Thach et al., 1991; St. John, 1996; St.-John & Paton, 2003). This type of breathing occurs as a result of very high respiratory drive and, as such, the air flow rate in gasp inspirations is very high in comparison to eupnoeic breaths (Thach et al., 1991).

Gasping does not appear to play a role in autoresuscitation from hypothermia-induced respiratory arrest in neonatal rats and hamsters (Corcoran & Milsom, 2003; Tattersall & Milsom, 2003). This is not surprising since there is considerable evidence to indicate that neonatal mammals do not suffer from hypoxia during hypothermia-induced respiratory arrest (Hill, 2000). It may be, however, that gasping in hypothermia does occur in older, and therefore larger, mammals due to the increased metabolic needs associated with their larger size. In support of this possibility, Adolph (1948a; 1961) reported “gasiing movements” in juvenile and adult rats upon re-warming from hypothermia in which respiratory arrest may or may not have occurred. In the current study the role of gasping in severe hypothermia and in autoresuscitation was examined in juvenile mammals. The last breath during cooling (before respiratory arrest) and the first breath upon re-warming (after respiratory arrest) were compared to the average
normal breath at 35°C and the average breath at the same $T_B$ during cooling as that at which the first breath occurred upon re-warming. Tidal volume of the last breath before respiratory arrest and the first breath after re-warming were significantly larger than normal breaths at 35°C (Table 2.2; Figure 2.10). However, these breaths were also of significantly longer duration indicating that they were not gasps since, as described above, gasping breaths are characterised by short $T_i$ in addition to large $V_T$ (Table 2.2; Figure 2.10). The air flow rate in both the last breath before arrest and the first breath upon re-warming was much lower than that of a normal breath indicating that, rather than gasps, these breaths were more like augmented breaths (Figure 2.10). Thus, in juvenile rats and hamsters gasping does not appear to play a role in either severe hypothermia prior to respiratory arrest or in autoresuscitation upon re-warming.

2.5.11. Is hypothermia a pathological state for neonatal and juvenile mammals?

Hypothermia is generally regarded as a pathological state for mammals. However, it may be that this is only true for those mammals that are normally homeothermic and endothermic ie. adult mammals. Neonatal rats and hamsters, while they do have a preferred $T_B$ are largely unable to thermoregulate before 12 days of age since they have limited thermogenic capacity, small body size and little insulation (Spiers & Adair, 1986; Blumberg, 1997; Blumberg & Sokoloff, 1998; Sokoloff et al., 2000). As a result neonatal rodents experience a wide range of $T_B$ in natural situations, depending on the presence or absence of the mother (Blumberg & Sokoloff, 1998; Rogalska & Caputa, 2004). Neonatal mammals survive extreme hypothermia, suffer no negative effects of low $T_B$ and if severe hypothermia results in respiratory arrest, neonates continue to meet their metabolic needs with adequate circulation and passive respiration through an open glottis (Adolph, 1951; Hill, 2000). If neonates are re-warmed from low $T_B$, breathing re-starts and they recover completely. It has been suggested that the amazing ability of neonatal mammals to tolerate low $T_B$ may be an adaptive trait enabling young mammals to conserve energy during times when the mother is absent (Adolph, 1951; Zimmer & Milsom, 2002; Tattersall & Milsom, 2003). Similarly, the ability of neonates to autoresuscitate may also be adaptive, enabling them to re-start breathing when the mother returns to the nest and re-warms them (Tattersall & Milsom, 2003). Therefore, very low body temperature may not be a pathological state for neonatal and juvenile mammals.
mammals and may instead reflect a metabolic choice similar to the metabolic choice made in hibernation or torpor (Hill, 2000).

2.5.12. What is the mechanism underlying the ability to autoresuscitate?
The ability to autoresuscitate from hypothermia-induced respiratory arrest appears to originate in the CRG for breathing in neonatal and juvenile mammals (Mellen et al., 2002; Milsom et al., 2002). One hypothesis for the underlying mechanism of the loss of the ability to autoresuscitate is that there is a shift in the relative roles of pacemaker and network properties of rhythm generation as mammals develop. The CRG in neonatal mammals appears to depend almost exclusively upon the endogenous rhythms of pacemaker cells located in the preBötzinger complex in the ventrolateral medulla since network properties (depending on reciprocal inhibition) are not required for rhythm generation in \textit{in vitro} preparations from neonatal mammals (Johnson et al., 1994; Ramirez & Richter, 1996; Brockhaus & Ballanyi, 1998). Pacemaker properties may play a different role in adult mammals since reciprocal inhibition is essential for rhythm generation in adult mammals \textit{in vivo} (Paton & Richter, 1995; Pierrefiche et al., 1998). The hybrid pacemaker-network model of central rhythm generation describes a developmental progression from a pacemaker driven CRG to one that is either dependent on network properties alone or is a hybrid of pacemaker and network properties working in conjunction with one another (Smith et al., 2000). It may be that the loss of the ability to autoresuscitate upon re-warming from hypothermia-induced respiratory arrest between the ages of 14 and 20 days of age in juvenile rats and 28 days and adulthood in hamsters reflects a shift in the relative importance of pacemaker and network properties of rhythm generation in these animals.

2.6. CONCLUSIONS
The ability to autoresuscitate was lost in rats between P14 and P20 and in hamsters between P28 and adulthood. The time scale over which the ability to autoresuscitate was lost was similar to the time scale reported for the maturation of thermogenesis and therefore the development of endothermy in these animals. During the initial stages of cooling \( f_R \) was observed to remain at euthermic levels until \( T_b \) fell to 16-25°C rather than declining linearly with \( T_b \). This indicates that juvenile rats and hamsters used thermogenesis during the initial stages of cooling in an attempt to return \( T_b \) to normal.
Finally, despite the fact that the metabolic rate of juvenile rats and hamsters is higher than that of neonates and that they are significantly larger, juveniles do not appear to suffer from hypoxia in severe hypothermia since gasping does not play a role in breathing at low $T_B$ in these animals.
3. THE ROLE OF PERSISTENT SODIUM CURRENTS IN RESPIRATORY RHYTHM GENERATION IN VITRO

3.1. INTRODUCTION
The _in vivo_ experiments described in the previous chapter demonstrated that there is a "window" in the development of juvenile rats and hamsters during which the ability to autoresuscitate is lost. In the case of rats, the window is narrowly defined between 14 and 18 days after birth. In contrast, hamsters appear to retain the ability to autoresuscitate until at least 28 days of age but lose it sometime before reaching adulthood. The loss of the ability to autoresuscitate may correspond to a change in central rhythm generation at that postnatal age, in particular, a shift in the relevant importance of pacemaker properties to network properties of rhythm generation. The objective of the following experiments was to investigate the mechanisms underlying the phenomenon of autoresuscitation and rhythm generation in neonatal mammals. The experiments were conducted upon neonatal rats and hamsters of ages well before the window. The brainstem-spinal cord (_en bloc_) preparation was used to allow direct manipulation of the environment of the rhythm generating region in the rostral ventrolateral medulla (RVLM) as well as direct recording from respiratory motor nerves.

3.1.1. The _in vitro_ neonatal mammal brainstem-spinal cord preparation
The brainstem-spinal cord preparation is one of many _in vitro_ preparations of neonatal mammals that produce a rhythmic discharge in respiratory motor nerves resembling respiratory activity in the intact animal (Suzue, 1984; Smith, 1991; Ballanyi _et al._, 1999; Richter & Spyer, 2001). The motor output produced by this preparation has been positively correlated with respiratory movements of the thorax and therefore is known as "fictive breathing" (Suzue, 1983; Suzue, 1984; Smith & Feldman, 1985; Smith & Feldman, 1987; Smith _et al._, 1990). The brainstem-spinal cord preparation includes the medulla and spinal cord, usually transected rostrally at the pontomedullary junction and caudally at the 5th-8th cervical nerve (C5-C8). It is known as the _en bloc_ (defined as "in assembly") preparation because the connections between many of the identified respiratory groups of neurons are preserved. The _en bloc_ preparation includes two
identified rhythm generating regions; the preBötzinger complex, a region both necessary and sufficient for the generation of respiratory rhythm, and also the parafacial respiratory group (Pre I area), a region recently identified that may interact with the preBötzinger complex to produce respiratory rhythm (Smith, 1991; Ballanyi et al., 1999; Onimaru & Homma, 2003; Zimmer, 2004). Synchronised respiratory related motor discharge can be recorded from the hypoglossal nerve (cranial nerve XII), the branches of the phrenic nerve (C4-C7) and other spinal nerves such as C1 (Smith et al., 1990; Jacquin et al., 1999).

The brainstem-spinal cord preparation is devoid of afferent inputs from the periphery and higher centres of the brain, however it retains central chemosensitivity (Paton et al., 1994; Ballantyne & Scheid, 2000; Lieske et al., 2000). The preparation reportedly contains both O₂ and CO₂/H⁺ chemoreceptors, primarily located in the rhythm generating region of the RVLM, which respond to hypocapnea, hypercapnea and hypoxia (Ballantyne & Scheid, 2000; Solomon et al., 2000). Exposure to acute hypercapnia or hypoxia produces an increase in fictive respiration in the en bloc preparation, as in intact animals (Brockhaus et al., 1993; Ballantyne & Scheid, 2000; Solomon et al., 2000; Feldman et al., 2003). Furthermore, if the lungs remain attached to the en bloc preparation, it is capable of responding to lung inflation with a response similar to the Hering-Breuer reflex (Ballanyi et al., 1999). Thus, many aspects of normal in vivo breathing are retained in this reduced preparation.

There is considerable controversy regarding the usefulness of reduced preparations, such as the brainstem-spinal cord, for studying respiration with the eventual intention of drawing conclusions about breathing in vivo. Of most concern is whether the motor output generated by the en bloc preparation corresponds to eupnea in the intact animal and specific questions therefore arise regarding the shape and frequency of the motor discharge. The en bloc preparation typically produces three patterns of motor output; incrementing/ augmenting, bell-shaped and decrementing as illustrated in Figure 3.1. Of these three, the most commonly expressed by preparations from rats and mice is the decrementing pattern of motor discharge (Onimaru et al., 1988; Smith et al., 1990; Di Pasquale et al., 1994; Zimmer, 2004). The decrementing burst shape is usually associated with gasping breaths in vivo whereas the incrementing and bell-shaped
Figure 3.1. Respiratory-like motor discharge patterns generated by hamster brainstem-spinal cord preparations at 27°C including A) incrementing or augmenting pattern, B) bell-shaped pattern and C) decrementing pattern.

burst patterns characterise eupnoeic breaths in in vivo adult mammals (Paton, 1996; St. John, 1996; St. John & Paton, 2003). This, taken with the observation that the frequency of bursting is significantly reduced in the en bloc preparation, when compared
to the intact animal, has led some authors to conclude that the in vitro brainstem-spinal cord preparation produces “fictive gasps” rather than “fictive breaths” (Paton, 1996; St. John, 1996; St. John & Paton, 2003). However, since otherwise intact but vagotomised neonatal rats produce decrementing motor discharge this pattern may instead arise as a result of vagotomy (Smith et al., 1990; Ballanyi et al., 1999). Similarly, the low frequency of discharge of the en bloc preparation, rather than indicating that the preparation is producing motor discharge similar to in vivo gasping, may also be a result of removal of mechanosensory inputs. Smith et al. (1990) reported that vagotomy in vivo resulted in a decline of breathing frequency from ~48 breaths/ min to ~14 breaths/ min, a frequency close to that exhibited by the en bloc preparation at 33-35°C (~15 bursts/ min). The low frequency of discharge in the en bloc preparation may also be a result of low temperature, since the preparation is typically maintained at 27°C, a temperature somewhat lower than euthermic Tb for neonatal rodents (33-35°C).

3.1.2. Pacemaker properties of central rhythm generation and Ina
Both inspiratory and pre-inspiratory pacemaker cells have been identified in the preBötzinger complex and parafacial respiratory group in neonatal mammals (Smith, 1991; Johnson et al., 1994; Onimaru et al., 1995; Onimaru & Homma, 2003). This information, when taken with the observation that synaptic transmission is not required for respiratory rhythm generation, has led to the conclusion that pacemaker properties are important, if not essential, for rhythm generation, at least in neonatal in vitro systems (Johnson et al., 1994; Shao & Feldman, 1997; Rekling & Feldman, 1998; Richter & Spyer, 2001). These observations concur with the prediction of the hybrid pacemaker-network model that the central rhythm generator (CRG) of neonatal mammals is primarily dependent upon pacemaker properties of rhythm generation (Smith et al., 2000).

The endogenous bursting of pacemaker cells in the RVLM depends on a number of ion conductances, the predicted roles of each in the generation of a burst of action potentials are shown in Figure 3.2. Between active phases the membrane of the pacemaker cell slowly depolarises as a result of inwardly rectifying K⁺ currents (Ikir) and non-specific cation currents (IH) flowing into the cell. At membrane potentials of ~60 mV, persistent Na⁺ channels (Nap) are activated leading to further depolarisation of the
membrane which results in the activation of T-type Ca\textsuperscript{2+} channels (Ca\textsubscript{T}) and the initiation of the active phase of bursting. The activation of persistent Na\textsuperscript{+} channels has the effect of amplifying subthreshold depolarisations, bringing the membrane to the activation threshold for fast Na\textsuperscript{+} channels, responsible for generating the large depolarisation of the first action potential (Onimaru et al., 1997; Ballanyi et al., 1999; Richter & Spyer, 2001). Once the membrane potential reaches the activation threshold for fast Na\textsuperscript{+} channels (Na\textsubscript{T}), multiple action potentials are produced.

**Figure 3.2.** Possible roles of ionic channels in the generation of bursting activity in an inspiratory pacemaker neuron. The approximate activation threshold of each channel is indicated by the position of the abbreviated channel name. The direction of change in membrane voltage (i.e. depolarising or hyperpolarising) as a result of the activation of each channel is indicated by the direction of the arrow. Resting membrane potential is indicated by the dashed line. Redrawn and compiled from: Onimaru et al. (1997); Ballanyi et al. (1999); Richter and Spyer (2001).
Each multi-spike burst is augmented by P-type, N-type and L-type Ca$^{2+}$ channels (Ca$_P$, Ca$_N$ and Ca$_L$) and is finally terminated by the activation of Ca$^{2+}$-activated K$^+$ channels (K$_{Ca}$) and inwardly rectifying K$^+$ channels (K$_{ir}$). The membrane remains hyperpolarised for a short time after each active phase before being slowly depolarised again. While each of these ion currents may be required for the normal function of RVLM pacemaker cells, the persistent sodium current (INa$_{p}$) is an essential element (Butera et al., 1999; Del Negro et al., 2002a). If INa$_{p}$ is blocked the majority of the pacemaker cells in the RVLM stop producing rhythmic bursts (Del Negro et al., 2001; Del Negro et al., 2002b; Parkis, 2002).

Recent studies have indicated that, while many respiratory pacemaker neurons are dependent upon INa$_{p}$ for intrinsic bursting, there is a subpopulation of pacemaker neurons that generate bursting independent of INa$_{p}$ (Thoby-Brisson & Ramirez, 2001; Parkis, 2002; Pena et al., 2004). The proportion of INa$_{p}$-dependent pacemaker cells and INa$_{p}$-independent cells in the preBötzingher complex changes over postnatal development in mice. Prior to 5 days after birth (P5) the vast majority of pacemaker cells in the preBötzingher complex of mice are dependent upon INa$_{p}$ for the generation of endogenous bursting (Pena et al., 2004). The proportion of cells that generate bursting in the absence of INa$_{p}$ increases between P5 and P15 while the proportion of INa$_{p}$-dependent pacemaker cells first decreases from P5 to P10 and then increases from P11 to P15 (Pena et al., 2004). In the following study, riluzole was used to block INa$_{p}$, and therefore the pacemaker properties of the majority of pacemaker cells, in the preBötzingher complex and elsewhere in the medulla, to determine the role of INa$_{p}$-dependent pacemaker properties in rhythm generation and in autoresuscitation from hypothermia-induced fictive respiratory arrest in neonatal rats and hamsters.

3.1.3. **Network properties of central rhythm generation and INa$_{p}$**

While synaptic transmission may not play an essential role in the rhythm generation of breathing in neonatal mammals, as it does in adults, synaptic mechanisms are most likely involved in the generation of breathing pattern in young animals. These network properties may also depend somewhat upon INa$_{p}$. Non-pacemaker respiratory neurons often generate a series of action potentials in response to a single depolarising event and the repetitive firing comprising a “network burst” appears to depend on INa$_{p}$. Once
the membrane of the cell is depolarised it remains depolarised for an extended period, generating multiple spikes as the membrane potential oscillates across the threshold for $\text{INa}_\text{p}$. It has been reported that $\text{INa}_\text{p}$ is important for bringing the membrane to threshold between these multiple spikes (Denac et al., 2000; Urbani & Belluzzi, 2000; Del Negro et al., 2002b). Blockade of $\text{INa}_\text{p}$ with riluzole can therefore result in a reduction in the number of spikes per burst and therefore in the duration of each burst (Urbani & Belluzzi, 2000; Del Negro et al., 2002b). Since network bursting properties are believed to be essential for the normal action of reciprocal inhibition, the oscillation in activity between different neurons or neuron groups, blockade of $\text{INa}_\text{p}$ could have profound effects on the pattern of inspiration and expiration and on the rhythm of breathing.

3.2. OBJECTIVE
The objective of these experiments was to determine if $\text{INa}_\text{p}$-dependent pacemaker properties were necessary for respiratory rhythm generation and/or autoresuscitation from hypothermia-induced fictive respiratory arrest in en bloc preparations from neonatal rats and hamsters (P0-4).
3.3. MATERIALS AND METHODS

3.3.1. Animals
Experiments were performed on 42 neonatal Sprague Dawley rats (*Rattus norvegicus*) and 56 neonatal Syrian hamsters (*Mesocricetus auratus*) aged 0-4 days old. Hamsters and rats were grouped according to age into P0-2 and P3-4 age groups for each species. All experiments were completed with the prior approval of the University of British Columbia Animal Care Committee acting under the guidelines of the Canadian Council for Animal Care. In the case of rats, the animals were provided by the University of British Columbia Animal Care Centre and delivered to the laboratory the morning of the experiment. In the case of hamsters, timed pregnant females (gestation day 8-12) were obtained from a commercial supplier (Charles River Laboratories, Canada) and maintained until delivery in the lab. Pregnant females were housed separately and provided with water and rodent chow *ad libitum*, supplemented with sunflower seeds and fruit. The exact time of birth was noted for accurate determination of the age of the neonates. Individual neonates were removed from the litter as needed for experiments.

3.3.2. The neonatal mammal in vitro brainstem-spinal cord preparation
The preparation used in these experiments was the brainstem-spinal cord preparation or *en bloc* preparation discussed in the Introduction. The brainstem-spinal cord was isolated using the technique outlined by Suzue (1984) and further described by a number of researchers (Onimaru, 1987; Smith, 1991; Zimmer & Milsom, 2002). Neonatal rats and hamsters, aged 0-4 days, were placed in a chamber and deeply anaesthetised with Halothane (4%). Once breathing ceased the animal was removed and immediately decerebrated with two lateral cuts at the level of bregma, each removing one half of the face. The remaining cerebral cortex was then removed from the braincase to complete decerebration. The body was then sectioned just below the rib cage and the remaining liver, heart, lungs and diaphragm removed. Skin and forelimbs were then removed and the preparation pinned dorsal side up in a dish filled with artificial cerebral spinal fluid (ACSF) at room temperature and equilibrated with 95% oxygen: 5% carbon dioxide to a pH of 7.4 [ACSF composed of (in mM): 113.0 sodium chloride, 3.0 potassium chloride, 1.2 sodium phosphate, 1.5 calcium chloride,
1.0 magnesium chloride, 30.0 sodium bicarbonate and 30.0 dextrose]. The muscles of the back and neck were removed to expose the vertebral column and braincase. Two lateral cuts were made in the caudal end of the vertebral column, one on each side of the spinal cord. The cuts were then extended, one vertebra at a time, until the skull was reached. Two lateral cuts were made rostral to caudal in the skull to meet the cuts on either side of the spinal cord. Lifting the skull up, the braincase was freed from the cerebellum and medulla and the flap of braincase and vertebral column removed. Once the cerebellum and spinal cord were completely exposed a midline incision was made in the cerebellum and each half removed with a lateral cut to fully reveal the medulla and pons. Starting again at the caudal end of the vertebral column the dorsal and ventral spinal nerve roots were cut and the spinal cord carefully lifted to free it from the vertebral column. In order to cut the cranial nerve roots from the ventral side of the brainstem the spinal cord was lifted and each cranial nerve was cut as close to the braincase as possible starting with those at the caudal end of the brainstem and moving to those more rostral. When the brainstem was completely freed from the braincase it was removed and transected at the pontomedullary junction and at the level of the 7th cervical root under a dissecting microscope. Finally, the meninges were removed and the preparation was pinned ventral side up in a small recording chamber (5 mL) of molded plexiglass, separated into two levels by a stainless steel grid (to provide maximum superfusion). ACSF bubbled with 5% carbon dioxide and 95% oxygen flowed through both levels of the chamber at a rate of 5-10 mL/ minute. The temperature of the fluid bathing the preparation was regulated by a Lauda refrigerating circulator (Ecoline RE-106) and monitored with a Physitemp (Model BAT-12, Type T) thermocouple submerged in the bathing fluid. At the start of the experiment the temperature of the bathing medium was 27°C.

3.3.3. Nerve recordings
Using a micromanipulator, a glass suction electrode (capillary tube with an inside diameter of 0.5 mm) filled with ACSF was positioned above the 1st cervical nerve root and/or the 4th cervical nerve root (Figure 3.3). Using suction, the nerve root was gently drawn into the electrode and a tight seal created between the nerve and electrode to permit recording of the motor nerve discharge. The nerve signal was amplified (50 000X), filtered (low pass 500 Hz; high pass 100 Hz) and recorded using DataQ
Instruments WindaqPro data acquisition software (Version 2.71) at a sampling rate of 1500 samples/second. During experiments, nerve activity was also monitored with an oscilloscope (Tektronix 5A18N dual Trace Amplifier), to provide a visual signal, and with an audio monitor (Grass AM8B) to provide an auditory signal.

**Figure 3.3.** *In vitro* experimental set-up showing the *in vitro* brainstem-spinal cord preparation (ventral view; transected rostrally at the pontomedullary junction and caudally at C7) in the recording chamber, the recording electrode and trace recording (raw and integrated). The bathing media, control ACSF and ACSF containing riluzole (ACSF+RIL), are indicated including the path of the fluid, indicated by black and grey arrows respectively. The peristaltic pump (P) and refrigerating circulator (RC) control ACSF temperature and flow rate. Flow through the system is controlled by way of three-way stopcocks (©).

**3.3.4. Drugs**

The endogenous rhythms of preBötzinger complex pacemaker cells, thought to generate breathing activity in neonatal mammals, are largely dependent upon the persistent sodium current in neonates younger than 5 days of age (Butera et al., 1999; Del Negro et al., 2002a; Rybak et al., 2003a; Pena et al., 2004). Urbani & Belluzi (2000) reported that the drug riluzole (2-amino-6-trifluoromethoxy benzothiazole) effectively
inhibited the persistent sodium current in the mammalian central nervous system in a dose-dependent fashion. Numerous authors have reported that riluzole effectively inhibits the endogenous rhythms of most respiratory pacemaker cells through its effects on \( \text{INa}_p \) (Del Negro et al., 2002b; Rybak et al., 2003b; Pena et al., 2004).

For the following experiments, a 2 mM stock solution of riluzole was created by dissolving the drug in a small amount of 1.0 M HCl which was then added to 50 mL distilled water. In experiments that required 200 \( \mu \text{M} \) riluzole, 1 mL of stock solution was added to 100 mL ACSF. For 20 \( \mu \text{M} \) riluzole, 0.2 mL stock was used in 100 mL ACSF. The pH of ACSF containing the solution of riluzole was 7.48.

The first experimental protocol, described below, was designed to determine the extent, and the rate, of the response to 20 \( \mu \text{M} \) and 200 \( \mu \text{M} \) riluzole of preparations from rats and hamsters (P0-4). A dose of 20 \( \mu \text{M} \) riluzole was selected for these experiments because this concentration of riluzole has been found to block the rhythmic bursting of \( \text{INa}_p \)-dependent pacemaker cells entirely in transverse slice preparations (Del Negro et al., 2002b; Koizumi & Smith, 2002; Parkis, 2002; Rybak et al., 2003b; Pena et al., 2004). The higher dose, 200 \( \mu \text{M} \) riluzole, was selected because this concentration of riluzole produced significant effects on motor output in under 10 minutes, the time between respiration arrest and autoresuscitation during the transitional cooling/rewarming protocol. In this well-established protocol the duration of respiratory arrest, or fictive respiratory arrest, at the minimum temperature is 10 minutes (Mellen et al., 2002; Zimmer & Milsom, 2002; Corcoran, 2003). In order to examine the role of \( \text{INa}_p \) in fictive autoresuscitation using the transitional cooling/rewarming protocol, riluzole was applied during the period of respiratory arrest, prior to re-warming from the minimum temperature, and a dose of riluzole was selected for these experiments that would produce significant effects during this period.

3.3.5. Experimental protocols

Riluzole (20 \( \mu \text{M} \) and 200 \( \mu \text{M} \)) at 27°C

Brainstem-spinal cords from P0-4 rats and hamsters were isolated at room temperature, as described above. The preparations were then placed in the recording chamber and superfused with ACSF at a temperature of 27°C. Control recordings were made for 20
minutes before riluzole was applied. The superfusate was then replaced with ACSF containing either 20 μM or 200 μM riluzole at the same temperature as control (Figure 3.3). When bursting had ceased, defined as 1 minute with no bursting, 6 mM potassium chloride (KCl) was applied through the bathing medium. Three minutes after bursting had ceased, the ACSF containing riluzole was replaced with regular ACSF. Washout of the drug continued for 10 minutes.

Transitional cooling/ re-warming: control
Brainstem-spinal cords from P0-2 and P3-4 rats and hamsters were isolated at room temperature, as described above. The preparations were then placed in the recording chamber and superfused with ACSF at a temperature of 27°C. The relatively long duration of experiments using the cooling/ re-warming protocol necessitated a shorter control period and therefore recordings were made at 27°C for 10 minutes before cooling commenced. It is important to note, however, that all preparations had reached a steady state, defined as relatively constant frequency of bursting and burst amplitude, prior to cooling. The ACSF bathing the brainstem-spinal cord was then cooled at a constant rate of 0.5°C / min until a temperature was reached at which bursting ceased. The superfusate was held at a temperature 1-2°C below the arrest temperature for 10 minutes, during which time no bursting activity occurred (the minimum temperature). If bursting resumed during this 10 minute period the temperature of the ACSF was further cooled until bursting ceased. At the end of 10 minutes of no bursting at the minimum temperature, the brainstem-spinal cord was then transitionally re-warmed at the same rate as it was cooled. When the temperature reached 27°C another 10 minutes of data were recorded before the experiment was terminated.

Transitional cooling/ re-warming: riluzole (200 μM)
Brainstem-spinal cords from P0-2 rats and hamsters were isolated at room temperature, as described above. The preparations were cooled and re-warmed according to the procedure outlined above for the control transitional cooling/ re-warming protocol. At the minimum temperature, 6 minutes after fictive respiratory arrest occurred and 4 minutes prior to re-warming, riluzole (200 μM) was bath applied in ACSF of the minimum temperature. The brainstem-spinal cord was then re-warmed at a constant rate of 0.5°C / min to 27°C. If bursting failed to resume upon re-warming, or failed during the re-
warming process, KCl (6 mM) was applied through the bathing medium at 27°C. A final 10 minutes of data were recorded at 27°C before the experiment was terminated.

3.3.6. Analysis
Raw nerve recordings were full wave rectified and integrated (100 samples/ bin; bin width=5 milliseconds) using DataQ Instruments Windaq Waveform Browser (Version 2.19) and Advanced Codas Calculation Package (Version 3.25). The respiratory-related motor nerve discharge was then analysed for frequency (bursts/ min), burst amplitude, burst duration and burst area. The data for burst amplitude and area were highly variable between preparations and were therefore normalised to control data from either before riluzole application (in the case of the drug protocol) or from 27°C at the start of the experiment (in the case of the cooling/ re-warming protocol).

In order to determine the effect of two doses of riluzole (20 and 200 μM) on brainstem-spinal cord preparations from rats and hamster aged 0-4 days, riluzole was applied at constant temperature and differences in burst frequency, amplitude, duration and area measured over time. A sample of 20 breaths was analysed from before drug application as the control measurement to which successive samples were compared. Samples of up to 20 breaths were taken at several points throughout the experiment until bursting could no longer be distinguished (either frequency or amplitude fell to zero). If the experiment was short (less than 10 minutes), or breathing frequency very low, the whole trace was analysed. Experiments were also conducted to determine if there was a differential response to the higher dose of riluzole (200 μM) between P0-2 and P3-4 rats and hamsters. The data were highly variable therefore to facilitate comparisons between age groups and species the data were normalised to relative time (TR). Samples at TR=0 were taken just prior to drug administration (control) and samples at TR=1.0 were taken at the point when bursting could no longer be distinguished, as a result of frequency or amplitude reaching zero. Samples of up to 20 breaths were taken at TR=0, 0.2, 0.4, 0.6 and 0.8.

In order to determine the effect of hypothermia on preparations from P0-2 and P3-4 rats and hamsters, en bloc preparations were cooled to a temperature at which fictive respiratory arrest occurred and then re-warmed. Up to 20 breaths were analysed for
burst frequency, amplitude, duration and area for each degree of cooling and re-warming. When burst frequency was low, each fictive breath was analysed. Burst amplitude and area were normalised to control measurements taken at 27°C prior to cooling.

To determine the effect of riluzole (200 μM) on the ability of en bloc preparations to autoresuscitate from hypothermia-induced fictive respiratory arrest, riluzole was applied at the minimum temperature to preparations from P0-2 rats and hamsters. As described above, up to 20 breaths were analysed for burst frequency, amplitude, duration and area for each degree during cooling, prior to application of riluzole, and each degree during re-warming, after riluzole application. When burst frequency was low, each fictive breath was analysed. Burst amplitude and area were normalised to control measurements taken at 27°C prior to cooling.

3.3.7. Statistics
Riluzole (20 μM and 200 μM) at 27°C
Amplitude and area data were normalised to control measurements taken at the beginning of each experiment and then arcsine transformed to render them normally distributed. Frequency and duration data were analysed in the raw state. Each of these four variables were analysed for 1) significant differences from control values taken prior to drug administration 2) significant differences in the response to 20 μM and 200 μM riluzole (P0-4 rats and hamsters) and 3) significant differences in the response of preparations from P0-2 and P3-4 neonates of each species to the higher dose of riluzole (200 μM).

Samples taken at several points in these experiments were compared to the control measurements for each variable (frequency, burst amplitude, duration and area) taken just prior to drug administration using a one way repeated measures ANOVA followed by a Bonferroni post hoc test. If the data were not normally distributed they were analysed using a repeated measures rank ANOVA followed by Dunn’s post hoc test. Each variable was analysed for significant differences in the response of preparations from P0-2 and P3-4 rats and hamsters to 200 μM riluzole using t-tests at each of Tr=0, 0.2, 0.4, 0.6, 0.8, 1.0. Finally, the total time required for bursting to cease after
application of riluzole was compared between 20 μM riluzole and 200 μM riluzole using a t-test for each species.

**Transitional cooling/re-warming: control**

Amplitude and area data were normalised to control measurements taken at the beginning of each experiment. Frequency and duration data were analysed in the raw state. The results were analysed for 1) significant differences from 27°C at the start of the experiment, and 2) significant differences between P0-2 and P3-4 age groups for each species. A one way repeated measures ANOVA followed by a Bonferroni post hoc test was used to determine if each variable at each degree during cooling and re-warming was significantly different from that variable at 27°C at the start of the experiment. If the data were not normally distributed they were analysed using a rank ANOVA followed by Dunn's post hoc test. P0-2 rats and hamsters were compared to P3-4 rats and hamsters using t-tests for each degree during cooling and re-warming to determine if there were significant differences within each species in the response of preparations of different ages to transitional cooling and re-warming.

**Transitional cooling/re-warming: riluzole (200 μM)**

Amplitude and area data were normalised to control measurements taken at the beginning of each experiment. Frequency and duration data were analysed in the raw state. The results were analysed for 1) significant differences from 27°C at the start of the experiment and 2) significant differences from values at that same temperature during cooling and re-warming in control experiments (in which brainstem-spinal cord preparations were not bathed in riluzole at the minimum temperature). A one way repeated measures ANOVA followed by a Bonferroni post hoc test was used to determine if each variable at each degree during cooling and re-warming was significantly different from that variable at 27°C at the start of the experiment. If the data were not normally distributed they were analysed using a rank ANOVA followed by Dunn's post hoc test. The values of frequency, amplitude, duration and area for those preparations that were bathed in riluzole (200 μM) at the minimum temperature and those that were not (control) were analysed for significant differences using t-tests at each degree during cooling and re-warming.
3.4. RESULTS

3.4.1. The effect of riluzole concentration on fictive breathing at 27°C

Riluzole of two concentrations (20 µM and 200 µM) was applied to brainstem-spinal cord preparations of neonatal rats and hamsters aged 0-4 days at a constant temperature of 27°C in order to determine the difference in response to these two doses of riluzole. Preparations from PO-4 rats responded to 20 µM riluzole with a decrease in the amplitude of the bursts of fictive breathing to zero in 46.6± 8.5 minutes and no change in the frequency of bursting until bursting could no longer be distinguished (Table 3.1; Figure 3.4; Figure 3.5). Preparations from P0-4 hamsters responded to 20 µM riluzole with a decrease in the frequency of bursts to zero in 12.9± 2.3 minutes and little change in burst amplitude (Table 3.1; Figure 3.4; Figure 3.6). If preparations from PO-4 rats and hamsters were superfused with normal ACSF and kept at a constant temperature of 27°C they continued to fictively breathe for 558± 161 and 617± 119 minutes, respectively (Corcoran and Milsom, unpublished data).

In both rats and hamsters the response to 200 µM riluzole was similar to that of 20 µM riluzole but it occurred more quickly (Figure 3.4; Figure 3.5; Figure 3.6). Preparations from rats ceased visible bursting 18.4± 2.7 minutes after application of 200 µM riluzole through a decline in burst amplitude to zero accompanied by a small decline in burst frequency. Hamster brainstem-spinal cords failed to generate fictive breathing in the presence of 200 µM riluzole significantly more quickly than those of rats, however, the failure occurred via a decline in the burst frequency to zero in 6.73± 0.97 minutes with only a slight decline in the amplitude of bursts (Table 3.1; Figure 3.5; Figure 3.6). While the amplitude of fictive breathing in hamster preparations did not decline significantly in the presence of riluzole, variability did increase after riluzole application. Riluzole (200 µM) blocked motor discharge entirely in preparations from P0-4 hamsters in less than 10 minutes. Preparations from rats continued to generate bursts for longer than 10 minutes in this concentration of riluzole. However, after 10 minutes of exposure to 200 µM riluzole, the effects on motor output of preparations from rats were significant. Therefore 200 µM riluzole was selected for use in the following experiments.
3.4.2. The effect of riluzole (200 μM) on fictive breathing in P0-2 and P3-4 rats and hamsters

In order to better compare the process of fictive respiratory failure in the presence of 200 μM riluzole as a function of postnatal age and species, the data for each variable were normalised to relative time (T\textsubscript{R}). Preparations from P0-2 and P3-4 neonatal rats failed to generate fictive breathing in the presence of riluzole (200 μM) through a decline in burst amplitude and area to zero with no significant effect on burst frequency or duration (Table 3.2; Figure 3.7). A significant decline in burst amplitude occurred in both P0-2 and P3-4 rats by T\textsubscript{R}=0.2 (reaching 59.8± 13.2% of control and 84.5± 5.7% of control, respectively). By T\textsubscript{R}=0.4, the burst area of preparations from P0-2 rats had declined to 44.1± 19.8% of control and burst area of preparations from P3-4 rats had declined to 57.9± 8.4% of control (Figure 3.7). The actual times corresponding to each T\textsubscript{R} are shown in Table 3.3 and Table 3.4.

Preparations from P0-2 and P3-4 hamsters also failed to generate bursting in the presence of 200 μM riluzole. Burst amplitude, duration and area were not significantly affected by riluzole in either age group (Table 3.2; Figure 3.8). Burst frequency, however, had decreased significantly from 9.6± 1.1 bursts/ min (control) to 5.4± 0.63 bursts/ min by T\textsubscript{R}=0.2 in preparations from P0-2 hamsters. By T\textsubscript{R}=0.6, frequency in preparations from P3-4 hamsters had also declined significantly from 8.3± 0.63 bursts/ min to 6.6± 0.89 bursts/ min (Figure 3.8).

Preparations from the P0-2 age group of both species appeared to respond to riluzole more quickly than those from the P3-4 age group. While there was no significant difference in the time to cessation of visible bursting after application of 200 μM riluzole (actual time corresponding to T\textsubscript{R}=1.0) between brainstems from P0-2 and P3-4 rats, the mean burst amplitude in P0-2 rats was significantly lower than P3-4 rats at T\textsubscript{R}=0.4 (59.8± 13.2% of control and 84.5± 5.7% of control, respectively; Table 3.2; Figure 3.7). The same trend occurred in hamsters in that the mean frequency of P0-2 hamsters at T\textsubscript{R}=0.2 was significantly lower than that of P3-4 hamsters at T\textsubscript{R}=0.2 (5.4± 0.63 and 8.1± 0.89 bursts/ min, respectively; Table 3.2; Figure 3.8).

Once visible bursting had ceased for 1 minute, KCl (6 mM) was applied to the
preparations through the bathing medium which increased [K⁺] in the ACSF to 9 mM. KCl failed to illicit a response in 100% of preparations from P0-2 and P3-4 rats however 85.7% of preparations from P0-2 and 57.1% of preparations from P3-4 hamsters generated rhythmic bursting after KCl was applied (Figure 3.4). A further 14.3% of P0-2 preparations and 28.6% of P3-4 preparations from hamsters generated some activity in response to KCl, though this activity was not rhythmic. Washout of the ACSF containing riluzole with regular ACSF did not restore fictive breathing in any preparations from rats or hamsters.

3.4.3. Effect of cooling to low temperature on fictive breathing in P0-2 and P3-4 rats and hamsters

Brainstem-spinal cords from P0-2 and P3-4 rats generated fictive breathing at 27°C at a frequency of 7.95±0.89 and 9.19±1.08 burst/ min, respectively (Table 3.5; Figure 3.9A). While there was a trend for older preparations to generate bursts more frequently there was no significant difference between the different age groups in rats. The frequency of bursting of brainstem-spinal cords from P0-2 hamsters at 27°C was 9.89±0.99 bursts/ min, significantly higher than that of preparations from P3-4 hamsters which burst at 7.25±0.70 bursts/ min at that temperature (Table 3.5; Figure 3.9B). There was no significant effect of species on the starting frequency of bursting in either age group. Bursts generated by preparations from P0-2 and P3-4 rats were significantly shorter in duration (0.90±0.09 sec and 0.82±0.04 sec, respectively) than those of both P0-2 and P3-4 hamsters (1.27±0.11 sec and 1.40±0.05 sec, respectively). Bursts from P3-4 hamsters were also significantly longer in duration than those of P0-2 hamsters (Table 3.5).

During cooling, the frequency of bursting decreased with temperature in a near linear fashion to zero (fictive respiratory arrest). The decline in burst frequency from control values at 27°C was significant by a temperature of 25°C in rats of both age groups and by 24°C in hamsters of each age group (Figure 3.9). Burst amplitude declined significantly from control values during initial cooling (27-22°C) in preparations from P3-4 rats but not in P0-2 rats or in hamsters (Figure 3.10). There was a corresponding significant increase in burst duration in preparations from P3-4 rats but not in P0-2 rats or in hamsters (Figure 3.10).
3.4.4. *Fictive respiratory arrest and autoresuscitation in PO-2 and P3-4 rats and hamsters*

Fictive respiratory arrest occurred at a mean temperature of 17.66± 1.18°C in brainstem-spinal cords from PO-2 rats and at 19.70± 1.15°C in P3-4 rats (Table 3.6). PO-2 hamsters arrested at a significantly lower mean temperature than rats of either age group, at 14.69± 0.79°C while P3-4 hamsters arrested at 17.52± 1.48°C. There was no significant effect of age on the temperature at which fictive respiratory arrest occurred in either rats or hamsters (Table 3.6).

During re-warming from the minimum temperature, 100% of preparations from rats and hamsters of both age groups autoresuscitated from fictive respiratory arrest (Figure 3.11; Figure 3.12). Preparations from PO-2 rats autoresuscitated at a mean temperature of 20.69± 1.2°C while those from P3-4 rats autoresuscitated at a mean temperature of 19.90± 2.2°C (Table 3.6). There was no significant difference between PO-2 and P3-4 rats in terms of the temperature at which preparations autoresuscitated. Preparations from PO-2 hamsters autoresuscitated at 16.07± 0.70°C, a significantly lower temperature than either age group of rats while those from P3-4 hamsters autoresuscitated 18.30± 1.0°C, a temperature not significantly different than rats or PO-2 hamsters (Table 3.6).

3.4.5. *Effect of re-warming from low temperature on fictive breathing in PO-2 and P3-4 rats and hamsters*

After autoresuscitation from fictive respiratory arrest at low temperatures, burst frequency increased in a near-linear fashion in preparations from PO-2 and P3-4 rats and hamsters (Figure 3.9). In all cases, burst frequency at 27°C at the end of the experiment was not significantly different than burst frequency at 27°C at the start of the experiment (Table 3.5; Figure 3.9). In these experiments there was no significant hysteresis in burst frequency; frequency was similar at a given temperature during cooling and re-warming. Burst amplitude during re-warming was significantly lower than control (27°C at start) in P3-4 rats and P3-4 hamsters but not in PO-2 rats and hamsters (Figure 3.10). There were, however, no significant differences between burst duration and area during cooling and burst duration and area during re-warming.
3.4.6. Effect of riluzole (200 μM) on autoresuscitation from fictive respiratory arrest in P0-2 rats and hamsters

All preparations from P0-2 rats autoresuscitated from fictive respiratory arrest upon re-warming in the presence of 200 μM riluzole (Figure 3.11). While there was no significant effect of riluzole on the temperature at which autoresuscitation occurred, there was a trend for preparations bathed in riluzole to autoresuscitate at higher temperatures than control preparations (23.56± 0.49°C and 20.29± 0.68°C, respectively; Table 3.8). In contrast, only 33% of preparations from P0-2 hamsters autoresuscitated in the presence of 200 μM riluzole. Of those that autoresuscitated, only 3 preparations continued rhythmic bursting throughout re-warming to 27°C (Figure 3.12). Those preparations from P0-2 hamsters that did autoresuscitate in the presence of riluzole did so at a mean temperature of 17.93± 0.93°C, a temperature not significantly different from control (Table 3.8).

3.4.7. Effect of riluzole (200 μM) on fictive breathing during re-warming in P0-2 rats and hamsters

After re-warming to 27°C, burst frequency in preparations from P0-2 rats that were bathed in riluzole at the minimum temperature was not significantly different from the burst frequency at 27°C prior to cooling (Table 3.7; Figure 3.13A). Similarly, those few preparations from hamsters that autoresuscitated and continued bursting throughout re-warming exhibited a mean burst frequency of 6.85± 3.31 burst/ min at 27°C, a frequency not significantly different than the starting frequency of 9.78± 0.67 burst/min (Table 3.7; Figure 3.13B). Burst amplitude during the latter part of re-warming had declined significantly from the amplitude at the start of the experiment in preparations from both P0-2 rats and P0-2 hamsters (Table 3.7; Figure 3.14). This decline in amplitude was also significantly reduced in both rats and hamsters in comparison to the amplitude of control preparations at those same temperatures during re-warming (Table 3.7; Figure 3.14). Similarly, there was a significant decline in burst duration and area in preparations from P0-2 rats during the latter part of re-warming that was not exhibited by control preparations at these temperatures (Table 3.7; Figure 3.14).

All preparations from hamsters either failed to autoresuscitate or failed to generate bursting before reaching 27°C or shortly thereafter. KCl (6 mM) was applied to these preparations once they reached 27°C and ceased bursting. Increasing [K⁺] to 9 mM
resulted in rhythmic bursting in 45.4% of PO-2 hamster preparations. A further 27.3% of PO-2 hamster preparations responded to KCl with either tonic discharge or non-rhythmic bursting.
Table 3.1. Time to the cessation of visible bursting in the absence of riluzole or after administration of riluzole (20 μM or 200 μM) in preparations from P0-4 rats and hamsters (*control data from Corcoran and Milsom, unpublished data). All data are reported as mean ± SEM. † denotes a significant difference between 20 μM riluzole and 200 μM riluzole and Ô denotes a significant difference between species (P<0.05)

<table>
<thead>
<tr>
<th>Species</th>
<th>Time to cessation of bursting (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control* (No riluzole)</td>
</tr>
<tr>
<td>Rat</td>
<td>558± 161</td>
</tr>
<tr>
<td>Hamster</td>
<td>617± 119</td>
</tr>
</tbody>
</table>
Figure 3.4. Sample recordings showing the effect of riluzole (20 μM and 200 μM) on motor output of brainstem-spinal cords from P0-4 rats and hamsters at 27°C, and the effect of KCl (6 mM) on motor output after bursting had ceased in the presence of riluzole.
Figure 3.5. The effect of two concentrations of riluzole, 20 µM (closed circles) and 200 µM (open squares), on A) the frequency and B) amplitude of fictive breathing in brainstem-spinal cord preparations from P0-4 rats at constant temperature (27°C). * denotes a significant difference from control values prior to administration of riluzole (P≤0.05)

Figure 3.6. The effect of two concentrations of riluzole, 20 µM (closed circles) and 200 µM (open squares), on A) the frequency and B) amplitude of fictive breathing in brainstem-spinal cord preparations from P0-4 hamsters at constant temperature (27°C). * denotes a significant difference from control values prior to administration of riluzole (P≤0.05)
Table 3.2. The effect of riluzole (200 μM) on the fictive respiratory output at 27°C (including burst frequency, amplitude, duration and area) in preparations from P0-2 and P3-4 rats and hamsters represented as a function of relative time (TR) where TR=0 is just prior to drug administration and TR=1.0 is when bursting ceased. All data are reported as mean ± SEM. * denotes a significant difference from control and # denotes a significant difference from the other age group (P≤0.05)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Species</th>
<th>Age</th>
<th>Relative Time (TR)</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td>0.2</td>
<td>0.4</td>
<td>0.6</td>
<td>0.8</td>
<td>1.0</td>
</tr>
<tr>
<td>Frequency (bursts/min)</td>
<td>Rat</td>
<td>P0-2</td>
<td>8.8±0.99</td>
<td>7.2±1.2</td>
<td>6.9±1.3</td>
<td>7.2±1.5</td>
<td>7.0±1.4</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P3-4</td>
<td>9.4±0.53</td>
<td>8.9±0.52</td>
<td>8.5±0.45</td>
<td>8.0±0.71</td>
<td>7.1±0.83</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Hamster</td>
<td>P0-2</td>
<td>9.6±1.1</td>
<td>5.4±0.63**</td>
<td>3.7±0.40**</td>
<td>2.9±0.54**</td>
<td>2.2±0.27*</td>
<td>0*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P3-4</td>
<td>8.3±0.63</td>
<td>8.1±0.89#</td>
<td>6.9±0.73#</td>
<td>6.6±0.89#</td>
<td>3.9±0.77*</td>
<td>0*</td>
</tr>
<tr>
<td>Burst amplitude (% of control)</td>
<td>Rat</td>
<td>P0-2</td>
<td>100</td>
<td>59.8±13.2*</td>
<td>23.3±2.6**</td>
<td>12.8±6.8*</td>
<td>2.02±1.1*</td>
<td>0*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P3-4</td>
<td>100</td>
<td>84.5±5.7*</td>
<td>63.3±9.7**</td>
<td>39.4±10.2*</td>
<td>14.8±6.5*</td>
<td>0*</td>
</tr>
<tr>
<td></td>
<td>Hamster</td>
<td>P0-2</td>
<td>100</td>
<td>101±2.8</td>
<td>95.7±2.2</td>
<td>91.9±5.7</td>
<td>85.8±5.3</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P3-4</td>
<td>100</td>
<td>100±1.6</td>
<td>96.7±3.6</td>
<td>98.8±4.6</td>
<td>92.3±7.6</td>
<td>-</td>
</tr>
<tr>
<td>Burst duration (sec)</td>
<td>Rat</td>
<td>P0-2</td>
<td>0.73±0.046</td>
<td>0.75±0.063</td>
<td>0.69±0.055</td>
<td>0.68±0.082</td>
<td>0.69±0.11</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P3-4</td>
<td>0.75±0.043</td>
<td>0.70±0.054</td>
<td>0.67±0.044</td>
<td>0.69±0.044</td>
<td>0.66±0.064</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Hamster</td>
<td>P0-2</td>
<td>1.39±0.21</td>
<td>1.54±0.21</td>
<td>1.51±0.12</td>
<td>1.53±0.12</td>
<td>1.38±0.16</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P3-4</td>
<td>1.40±0.07</td>
<td>1.36±0.08</td>
<td>1.39±0.07</td>
<td>1.40±0.09</td>
<td>1.36±0.08</td>
<td>-</td>
</tr>
<tr>
<td>Burst area (% of control)</td>
<td>Rat</td>
<td>P0-2</td>
<td>100</td>
<td>85.3±9.6</td>
<td>44.1±19.8*</td>
<td>30.6±17.2*</td>
<td>13.5±6.5*</td>
<td>0*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P3-4</td>
<td>100</td>
<td>73.4±10.1</td>
<td>57.9±8.4*</td>
<td>48.0±11.7*</td>
<td>21.3±11.1*</td>
<td>0*</td>
</tr>
<tr>
<td></td>
<td>Hamster</td>
<td>P0-2</td>
<td>100</td>
<td>124±12.3</td>
<td>129.6±17.6</td>
<td>83.3±8.12</td>
<td>79.1±15.4</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P3-4</td>
<td>100</td>
<td>98.7±3.63</td>
<td>100.1±6.78</td>
<td>93.4±6.19</td>
<td>88.2±7.01</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 3.7. The effect of riluzole (200 μM) on A) fictive breathing frequency B) burst amplitude C) duration and D) area, over relative time (TR) where TR=0 was just prior to drug administration and TR=1.0 was when bursting ceased, in brainstem-spinal cord preparations from P0-2 (closed circles) and P3-4 (open squares) rats at constant temperature (27°C). * denotes a significant difference from control values at TR=0 and # denotes a significant difference from the other age group (P≤0.05)
Figure 3.8. The effect of riluzole (200 µM) on A) fictive breathing frequency B) burst amplitude C) duration and D) area, over relative time (TR) where TR=0 was just prior to drug administration and TR=1.0 was when bursting ceased, in brainstem-spinal cord preparations from P0-2 (closed circles) and P3-4 (open squares) hamsters at constant temperature (27°C). * denotes a significant difference from control values at TR=0 and # denotes a significant difference from the other age group (P≤0.05)
**Table 3.3.** Actual time corresponding to $T_R$, where $T_R=0$ was just prior to administration of 200 μM riluzole and $T_R=1.0$ was when bursting ceased in preparations from PO-2 and P3-4 rats. All data are reported as mean ± SEM.

<table>
<thead>
<tr>
<th>Actual time corresponding to $T_R$ (minutes)</th>
<th>0</th>
<th>0.2</th>
<th>0.4</th>
<th>0.6</th>
<th>0.8</th>
<th>1.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>PO-2</td>
<td>0</td>
<td>3.93±0.78</td>
<td>7.85±1.56</td>
<td>11.78±2.34</td>
<td>15.71±3.12</td>
<td>19.64±3.90</td>
</tr>
<tr>
<td>P3-4</td>
<td>0</td>
<td>3.47±0.79</td>
<td>6.95±1.59</td>
<td>10.42±2.38</td>
<td>13.89±3.17</td>
<td>17.36±3.97</td>
</tr>
</tbody>
</table>

**Table 3.4.** Actual time corresponding to $T_R$, where $T_R=0$ was just prior to administration of 200 μM riluzole and $T_R=1.0$ was when bursting ceased in preparations from PO-2 and P3-4 hamsters. All data are reported as mean ± SEM.

<table>
<thead>
<tr>
<th>Actual time corresponding to $T_R$ (minutes)</th>
<th>0</th>
<th>0.2</th>
<th>0.4</th>
<th>0.6</th>
<th>0.8</th>
<th>1.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>PO-2</td>
<td>0</td>
<td>1.68±0.28</td>
<td>3.37±0.56</td>
<td>5.05±0.83</td>
<td>6.74±1.1</td>
<td>8.42±1.4</td>
</tr>
<tr>
<td>P3-4</td>
<td>0</td>
<td>1.01±0.22</td>
<td>2.02±0.44</td>
<td>3.03±0.66</td>
<td>4.04±0.88</td>
<td>5.05±1.1</td>
</tr>
</tbody>
</table>
Table 3.5. The effect of age and species on the frequency, amplitude, duration and area of fictive respiratory output prior to cooling (27°C start) and after re-warming (27°C end) in en bloc preparations from P0-2 and P3-4 rats and hamsters. All data are reported as mean ± SEM. Sample size is given in brackets for each treatment. * denotes a significant difference from 27°C prior to cooling and † denotes a significant difference from the other age group (P<0.05). Sample size is shown in brackets.

<table>
<thead>
<tr>
<th></th>
<th>Age</th>
<th>RAT P0-2</th>
<th>RAT P3-4</th>
<th>HAMSTER P0-2</th>
<th>HAMSTER P3-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency (bursts/ min)</td>
<td>27°C Start</td>
<td>7.95± 0.89 (8)</td>
<td>9.19± 1.08 (7)</td>
<td>9.89± 0.99 (8)*</td>
<td>7.25± 0.70 (8)†</td>
</tr>
<tr>
<td></td>
<td>27°C End</td>
<td>7.19± 0.95 (8)</td>
<td>8.91± 1.09 (7)</td>
<td>10.00± 1.1 (8)*</td>
<td>6.32± 0.70 (8)*</td>
</tr>
<tr>
<td>Amplitude (% of control)</td>
<td>27°C Start</td>
<td>100 (8)</td>
<td>100 (7)</td>
<td>100 (8)</td>
<td>100 (8)</td>
</tr>
<tr>
<td></td>
<td>27°C End</td>
<td>84.5± 11.7 (8)</td>
<td>69.3± 9.3 (7)*</td>
<td>86.9± 3.2 (8)*</td>
<td>70.6± 8.2 (8)*</td>
</tr>
<tr>
<td>Duration (sec)</td>
<td>27°C Start</td>
<td>0.90± 0.09 (8)</td>
<td>0.82± 0.04 (7)</td>
<td>1.27± 0.11 (8)</td>
<td>1.40± 0.053 (8)</td>
</tr>
<tr>
<td></td>
<td>27°C End</td>
<td>1.07± 0.06 (8)†</td>
<td>0.78± 0.05 (7)*</td>
<td>1.21± 0.22 (8)</td>
<td>1.58± 0.10 (8)</td>
</tr>
<tr>
<td>Area (% of control)</td>
<td>27°C Start</td>
<td>100 (8)</td>
<td>100 (7)</td>
<td>100 (8)</td>
<td>100 (8)</td>
</tr>
<tr>
<td></td>
<td>27°C End</td>
<td>110.6± 7.2 (8)†</td>
<td>70.9± 7.9 (7)*</td>
<td>84.1± 7.6 (8)</td>
<td>75.6± 20.1 (8)</td>
</tr>
</tbody>
</table>

Table 3.6. The effect of age on temperature of fictive respiratory arrest and autoresuscitation upon re-warming in preparations from P0-2 and P3-4 rats and hamsters. All data are reported as means ± SEM. 0° denotes a significant difference from rats of both age groups. Sample size is shown in brackets.

<table>
<thead>
<tr>
<th>Age</th>
<th>Temperature of arrest (°C)</th>
<th>Temperature of autoresuscitation (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P0-2</td>
<td>17.66± 1.18 (8)</td>
<td>20.69± 1.16 (8)</td>
</tr>
<tr>
<td>P3-4</td>
<td>19.70± 1.15 (7)</td>
<td>19.90± 2.20 (7)</td>
</tr>
<tr>
<td>Hamster</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P0-2</td>
<td>14.69± 0.79° (8)</td>
<td>16.07± 0.70° (8)</td>
</tr>
<tr>
<td>P3-4</td>
<td>17.52± 1.48 (8)</td>
<td>18.30± 1.0 (8)</td>
</tr>
</tbody>
</table>

76
Figure 3.9. The effect of transitional cooling and re-warming on fictive breathing frequency in brainstem-spinal cord preparations of A) rats and B) hamsters aged P0-2 (solid circles) and P3-4 (open squares). * denotes a significant difference from control (27°C at start of experiment) and # denotes a significant difference from P0-2 age group at that temperature (P≤0.05)
Figure 3.10. The effect of transitional cooling and re-warming on burst amplitude, duration and area in brainstem-spinal cord preparations of A) rats and B) hamsters aged P0-2 (solid circles) and P3-4 (open squares). * denotes a significant difference from control (27°C at start of experiment) and # denotes a significant difference from P0-2 age group at that temperature (P≤0.05).
Figure 3.11. Sample recordings of motor output from brainstem-spinal cord preparations of rats (P0-2) showing A) the effect of transitional cooling and re-warming on motor output and B) the effect of riluzole (200 μM) administered at the minimum temperature on the ability to autoresuscitate from hypothermia-induced fictive respiratory arrest.
Figure 3.12. Sample recordings of motor output from brainstem-spinal cord preparations of hamsters (P0-2) showing the effect of transitional cooling and re-warming on motor output and the effect of riluzole (200 μM) on the ability to autoresuscitate from hypothermia-induced fictive respiratory arrest.
Table 3.7. The frequency, amplitude, duration and area of fictive respiratory output prior to cooling (27°C start) and after re-warming (27°C end) of en bloc preparations from PO-2 rats and hamsters that were either bathed in riluzole (riluzole 200 µM) or not bathed in riluzole (control) prior to re-warming. All data are reported as mean ± SEM. Sample size is given in brackets for each treatment. * denotes a significant difference from 27°C prior to cooling and † denotes a significant difference from the control group (P≤0.05). Sample size is shown in brackets.

<table>
<thead>
<tr>
<th></th>
<th>RAT</th>
<th>HAMSTER</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Control</td>
</tr>
<tr>
<td><strong>Frequency (bursts/ min)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>27°C Start</td>
<td>7.95± 0.89 (8)</td>
<td>7.79± 1.01 (8)</td>
</tr>
<tr>
<td>27°C End</td>
<td>7.19± 0.95 (8)</td>
<td>7.67± 1.2 (8)</td>
</tr>
<tr>
<td><strong>Amplitude (% of control)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>27°C Start</td>
<td>100 (8)</td>
<td>100 (8)</td>
</tr>
<tr>
<td>27°C End</td>
<td>84.5± 11.7 (8)</td>
<td>53.3± 4.4 (8)</td>
</tr>
<tr>
<td><strong>Duration (sec)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>27°C Start</td>
<td>0.90± 0.09 (8)</td>
<td>0.86± 0.08 (8)</td>
</tr>
<tr>
<td>27°C End</td>
<td>1.07± 0.06 (8)</td>
<td>0.68± 0.07 (8)</td>
</tr>
<tr>
<td><strong>Area (% of control)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>27°C Start</td>
<td>100 (8)</td>
<td>100 (8)</td>
</tr>
<tr>
<td>27°C End</td>
<td>110.6± 7.2 (8)</td>
<td>61.7± 8.3 (8)</td>
</tr>
</tbody>
</table>

Table 3.8. The temperature of fictive respiratory arrest and autoresuscitation upon re-warming of preparations from PO-2 rats and hamsters including those bathed in riluzole (riluzole 200 µM) and those not bathed in riluzole (control) prior to re-warming. All data are reported as mean ± SEM. † denotes a significant difference from the control group (P≤0.05). Sample size is shown in brackets.

<table>
<thead>
<tr>
<th></th>
<th>Temperature of arrest (°C)</th>
<th>Temperature of autoresuscitation (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>17.66± 1.18 (8)</td>
<td>20.69± 1.16 (8)</td>
</tr>
<tr>
<td>Riluzole (200 µM)</td>
<td>20.65± 0.34 (8)</td>
<td>23.56± 0.49 (8)</td>
</tr>
<tr>
<td>Hamster</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>14.69± 0.79 (8)</td>
<td>16.07± 0.70 (8)</td>
</tr>
<tr>
<td>Riluzole (200 µM)</td>
<td>17.23± 0.80 (12)</td>
<td>17.93± 0.93 (4)</td>
</tr>
</tbody>
</table>
Figure 3.13. The effect of transitional cooling and re-warming on fictive breathing frequency in brainstem-spinal cord preparations of A) P0-2 rats and B) P0-2 hamsters that were either bathed in 200 μM riluzole (open squares) or not bathed in riluzole (solid circles) at the minimum temperature prior to re-warming. Note that only four P0-2 hamsters autoresuscitated upon re-warming in riluzole. * denotes a significant difference from control (27°C at start of experiment) and † denotes a significant difference from control group (P≤0.05).
Figure 3.14. The effect of transitional cooling and re-warming on burst amplitude, duration and area in brainstem-spinal cord preparations of A) PO-2 rats and B) PO-2 hamsters that were either bathed in 200 μM riluzole (open squares) or not bathed in riluzole (solid circles) at the minimum temperature prior to re-warming. Note that only four PO-2 hamsters autoresuscitated upon re-warming in riluzole. * denotes a significant difference from control (27°C at start of experiment) and † denotes a significant difference from control group (P ≤ 0.05).
3.5. DISCUSSION

As mammals develop, the roles of intrinsic properties of respiratory neurons (pacemaker properties) and synaptic connections between respiratory neurons (network properties) are hypothesised to undergo a shift in relevance. Pacemaker properties of respiratory rhythm generation are believed to be both necessary and sufficient for normal breathing activity in neonatal mammals in vitro (Pena et al., 2004; Smith et al., 2004). Thus the CRG for breathing of neonatal mammals is not dependent upon synaptic transmission whereas the CRG of adult mammals requires synaptic mechanisms for the generation of breathing (Hayashi & Lipski, 1992; Johnson et al., 1994; Onimaru et al., 1995; Shao & Feldman, 1997; Pierrefiche et al., 1998; Thoby-Brisson et al., 2000). The endogenous rhythms of pacemaker cells in the RVLM of neonatal rats and mice prior to 5 days of age are primarily dependent upon $I_{Na_p}$ (Butera et al., 1999; Del Negro et al., 2002a; Pena et al., 2004). In the current study, $I_{Na_p}$-dependent pacemaker properties were blocked with the drug riluzole to determine the role of $I_{Na_p}$ in central rhythm generation in rats and hamsters and to determine if $I_{Na_p}$-dependent properties are required for autoresuscitation from hypothermia-induced fictive respiratory arrest.

3.5.1. Effect of riluzole concentration on fictive breathing at 27°C

At the start of the current experiments, the effects of two concentrations of riluzole on fictive breathing were determined in order to select an appropriate dose of riluzole for use with the en bloc preparation of neonatal rats and hamsters. Riluzole has often been used to block $I_{Na_p}$ in brainstem transverse slice preparations from neonatal rats and mice and therefore the appropriate dose is well established for this preparation. For these thin slices (400-700 $\mu$m in cross section) concentrations of 10-50 $\mu$M riluzole, administered in the bathing medium, are adequate to block rhythmic bursting in most $I_{Na_p}$-dependent pacemaker cells in the RVLM (Del Negro et al., 2002b; Koizumi & Smith, 2002; Parkis, 2002; Rybak et al., 2003b; Pena et al., 2004). The en bloc preparation is much larger than transverse slices (~4 mm in cross section) and diffusion of riluzole into the rhythm generating regions may be limited. Therefore, two concentrations of riluzole were selected for the current study; 20 $\mu$M, which has been shown to block $I_{Na_p}$-dependent pacemaker properties in transverse slice preparations...
and a higher dose of 200 μM, to insure adequate diffusion into the brainstem in the larger en bloc preparation.

In order to test the role of INa_p in fictive autoresuscitation using the transitional cooling/re-warming protocol riluzole was applied during the period of respiratory arrest (10 minutes) at the minimum temperature prior to re-warming. Therefore, a dose of riluzole had to be determined in the current study that caused significant effects on motor output in less than 10 minutes. The higher dose of riluzole tested (200 μM) produced significant effects on motor output, not qualitatively different from the effects of 20 μM riluzole, in both rats and hamsters in less than 10 minutes (Figure 3.5; Figure 3.6). Therefore, 200 μM riluzole was selected for use in the transitional cooling/re-warming protocol. In order that the results be comparable, this dose of riluzole was also used to examine the differences between P0-2 and P3-4 rats and hamsters in terms of the response to riluzole at 27°C. No information is available on the rate of diffusion of riluzole into the interstitial spaces between brainstem neurons or on the mechanisms by which riluzole binds to Na^+ channels. Therefore, the precise dose of riluzole experienced by the target cells in the RVLM, located 300-600 μm from the ventral surface of the medulla, could not be determined in the current study.

3.5.2. Effect of riluzole (200 μM) on fictive breathing at 27°C: species differences
Preparations from rats and hamsters behaved very differently after the application 200 μM riluzole. Preparations from hamsters reacted more quickly to riluzole than those from rats, and also failed sooner after exposure to riluzole (Table 3.1; Figure 3.5; Figure 3.6). As such, the data were normalised to relative time (TR) in order to compare the extent of the response to riluzole between rats and hamsters, and between age groups of each species, independent of the rate of response (Figure 3.7; Figure 3.8). Preparations from rats responded to riluzole with an almost immediate decline in the amplitude and area of each burst whereas preparations from hamsters responded with an almost immediate decline in the frequency of bursting. This implies a very different mechanism of effect of riluzole in the two species.

The endogenous bursting behaviour of most pacemaker cells in the RVLM is made possible by INa_p which brings the membrane to threshold for INa_t that in turn gives rise to
the action potentials that comprise each burst (Figure 3.2). In preparations from neonatal rats of both age groups the frequency of bursting was not significantly altered by the addition of riluzole, a drug known to effectively block \( I_{\text{Na}_p} \) (Figure 3.7). Similar observations have been made by Pena et al. (2004) and Del Negro et al. (2002b) who reported that the frequency of bursting of preparations from rats and mice remained unaltered after the application of riluzole (1-200 \( \mu \text{M} \)). It was hypothesised at the start of the current study that \( I_{\text{Na}_p} \)-dependent pacemaker properties were essential for central rhythm generation in both rats and hamsters but the observations appear to refute this hypothesis. However, there are a number of possible interpretations of the observed lack of effect of riluzole upon the rhythm of fictive breathing. To begin with, since intracellular or extracellular recordings were not made in the current study, it is possible that riluzole failed to block \( I_{\text{Na}_p} \) in \textit{en bloc} preparations from rats and that \( I_{\text{Na}_p} \)-dependent endogenous rhythms continued to be generated in the presence of the drug. Alternatively, it is possible that riluzole blocked \( I_{\text{Na}_p} \) but that this current was not necessary for the pacemaker properties of the rhythm-generating neurons, or at least not necessary for the pacemaker properties of \textit{all} the rhythm-generating neurons. In answer to these first two possibilities, it is has been reported by many authors that \( I_{\text{Na}_p} \) is effectively blocked by riluzole and that the rhythmic bursting of the majority of pacemaker cells in the RVLM of P0-5 rats ceases in the presence of riluzole (Urbani & Belluzzi, 2000; Del Negro et al., 2001; Del Negro et al., 2002b; Parkis, 2002). However, it is likely that some pacemaker neurons continued to generate rhythmic bursting in the presence of riluzole since not all pacemaker cells are dependent upon \( I_{\text{Na}_p} \). As Pena et al. (2004) have demonstrated, there is a sub-population of pacemaker neurons, located in the RVLM of neonatal mice, that are insensitive to riluzole, indicating that the pacemaker properties of these cells do not depend upon \( I_{\text{Na}_p} \). Since the majority of these riluzole-insensitive cells fail to generate rhythmic bursting in the presence of cadmium (a known antagonist of calcium currents), the pacemaker properties of this sub-population are assumed to depend upon calcium currents (Pena et al., 2004). It appears, however, that the endogenous bursting of these calcium-dependent pacemakers is not directly sustained by voltage-activated calcium channels, but instead by a calcium-activated non-specific cation current (Pena et al., 2004). These calcium-dependent, riluzole-insensitive pacemakers are few in number in animals younger than 5 days, however, they may be sufficient to produce rhythmic motor output after
blockade of the riluzole-sensitive cells in neonatal rats (Del Negro et al., 2002b; Pena et al., 2004). When both populations of pacemaker cells are blocked simultaneously, motor output in preparations from neonatal mice has been shown to fail (Pena et al., 2004).

While riluzole appeared to have no significant effect on the generation of the rhythm of fictive breathing in rats this drug caused significant changes in the pattern of fictive breathing. Riluzole (200 μM) produced almost immediate and significant declines in burst amplitude and area in preparations from both P0-2 and P3-4 rats (Figure 3.7). Many authors have reported dose-dependent declines in burst amplitude and area, similar to those we observed, in response to 1-200 μM riluzole (Del Negro et al., 2002b; Koizumi & Smith, 2002; Abdala et al., 2004). Declines in burst amplitude and area, in the absence of significant effects on frequency of bursting, imply that riluzole produced effects other than the known effects on \( \text{I}_{\text{Na}_p} \)-dependent pacemaker activity.

The effects of riluzole upon the \( \text{I}_{\text{Na}_p} \)-dependent bursting properties of respiratory pacemaker neurons have been relatively well established (Del Negro et al., 2002a; Del Negro et al., 2002b; Rybak et al., 2003b; Pena et al., 2004; Smith et al., 2004). However, this drug has also been shown to affect fast/ transient sodium currents (\( \text{I}_{\text{Na}} \)), which are responsible for the large depolarisation of the action potential, and high voltage-activated calcium currents, which are involved in the release of glutamate, an excitatory neurotransmitter (Huang et al., 1997; Urbani & Belluzzi, 2000; Del Negro et al., 2002a). These calcium and fast sodium currents are much less sensitive to riluzole than \( \text{I}_{\text{Na}_p} \) resulting in negligible effects at low doses but possibly significant effects at high doses (Urbani & Belluzzi, 2000; Del Negro et al., 2002a). Urbani & Belluzzi (2000) demonstrated that 100 μM riluzole caused a significant shift in the steady state inactivation curve of \( \text{I}_{\text{Na}_r} \) in cortical neurons, however, it is unknown what effect such a shift would have upon the bursting properties of respiratory neurons (Urbani & Belluzzi, 2000). However, a reduction in the size of the action potentials produced by respiratory-related pre-motor and motor neurons could result in the decline in the amplitude of motor discharge observed in the current study.
While it is possible that riluzole altered INaᵣ in the current study it has been often reported that riluzole preferentially blocks INaᵦ, even though INaᵦ and INaᵣ are likely sustained by the same type of channel (Denac et al., 2000; Urbani & Belluzzi, 2000). So, alternatively, the effect of riluzole on motor discharge in en bloc preparations from rats could have been caused by effects on network bursting properties of non-pacemaker cells. Non-pacemaker neurons in the RVLM often respond to a single depolarising event with a burst of action potentials and INaᵦ may be important for bringing the membrane to threshold between the multiple spikes of each burst (Denac et al., 2000; Urbani & Belluzzi, 2000; Del Negro et al., 2002b). Blockade of INaᵦ may result in a reduction in the number of spikes per burst and therefore in the duration and shape of each burst (Urbani & Belluzzi, 2000; Del Negro et al., 2002b). Since network bursting properties are believed to be essential for the normal action of reciprocal inhibition, the oscillation in activity between different neurons or neurons groups, blockade of INaᵦ could have profound effects on the pattern of inspiratory and expiratory activity and also on the rhythm of breathing. Thus, the decline in burst amplitude and area observed in preparations from rats in the current study could have been caused by the effects of riluzole upon network bursting properties important for the generation of breathing pattern.

Preparations from hamsters of both age groups (P0-2 and P3-4) reacted to the application of riluzole with an abrupt decline in the frequency of fictive breathing with no significant change in the pattern of the bursts (Figure 3.8). While no previous results exist for hamsters, alterations in the frequency of bursting in response to riluzole have been reported in preparations from rats (Koizumi & Smith, 2002; Rybak et al., 2003b; Smith et al., 2004). However, the decline in the frequency of fictive breathing observed by these authors was always preceded and accompanied by declines in burst amplitude and area. It is worth noting that these results appear to be in direct conflict with those described above for rats and mice in which riluzole reportedly produced no alterations in burst frequency and only produced significant effects in burst pattern (Del Negro et al., 2002b; Pena et al., 2004). In any case, no such declines in burst amplitude, duration or area were observed in preparations from hamsters in the current study (Figure 3.8). Thus, while riluzole appeared to affect the rhythm generation of fictive breathing in hamsters, it had no significant effect on pattern generation (as indicated by burst
amplitude, duration and area) as it did in rats. Admittedly, riluzole may act on Ina_p-dependent pattern generation in hamster preparations with different dose-dependence than in rat preparations. However, the results do imply that Ina_p-dependent network-bursting properties are of less importance to the generation of pattern in hamsters than in rats.

Strangely, washout of the ACSF containing riluzole with normal ACSF did not enable fictive breathing to return in any of the preparations studied. This was unexpected since the effects of riluzole on persistent Na^+ channels are reportedly reversible (Hebert et al. 1994). Riluzole reversibly binds to the alpha subunit of closed (inactive) voltage-dependent sodium channels (Hebert et al. 1994). Persistent Na^+ channels may or may not have the same structure as fast/ transient Na^+ channels however riluzole preferentially binds to persistent Na^+ channels (Urbani & Belluzzi, 2000. Unfortunately there is no information available regarding the effects of washout of riluzole in in vitro preparations. It may be that that blockade of Ina_p in en bloc preparations produced other effects which inhibited fictive breathing even after riluzole was washed out. Alternatively, the other potential effects of riluzole, as discussed above, could inhibit fictive breathing even after Ina_p has resumed.

Interestingly, once fictive respiratory failure had occurred in the presence of riluzole in preparations from hamsters the application of KCl to the bathing media frequently initiated rhythmic bursting again for a short while (Figure 3.4). The extracellular concentration of potassium (K^+) is well known to be an important contributor to the state of excitation of respiratory-related neurons (Rybak et al., 2003b; Tryba et al., 2003). The [K^+] in normal ACSF in the current study was physiological at 3 mM however the addition of KCl increased the [K^+] to 9 mM. The addition of K^+ likely caused the membranes of respiratory-related neurons to depolarise which could have activated Ina_f and produced a series of action potentials. This indicates that the CRG of hamsters was still capable of generating rhythmic fictive breathing, even after fictive respiratory arrest in the presence of riluzole.

As discussed in the introduction, it has been hypothesised that pacemaker properties are essential for rhythm generation in neonatal mammals (Smith et al., 2000; Del Negro 89
et al., 2001; Richter & Spyer, 2001). As a result of modelling studies and experimental work \( I_{\text{NaP}} \) has been implicated as a current that is essential to most pacemaker properties in the RVLM (Butera et al., 1999; Del Negro et al., 2002a; Rybak et al., 2003b; Koizumi & Smith, 2004; Pena et al., 2004). The results of the current study regarding preparations from hamsters support the hypothesis that \( I_{\text{NaP}} \)-dependent pacemaker properties are essential to respiratory rhythm generation in neonatal mammals. However, the results from preparations from rats instead provide evidence that \( I_{\text{NaP}} \) plays an essential role in network bursting properties in this species.

3.5.3. Effect of riluzole (200 \( \mu \text{M} \)) on fictive breathing at 27°C: developmental influences

As discussed above, there were significant differences in the response of preparations from rats and hamsters to riluzole. Until now, the two age groups of each species have been discussed together, in order to simplify interspecies comparisons. There were, however, some small differences observed between preparations from P0-2 and P3-4 rats and hamsters. The amplitude and area of motor output from preparations of P0-2 rats, and the frequency of bursting in preparations from P0-2 hamsters, declined more quickly than those of P3-4 rats and hamsters (Figure 3.7; Figure 3.8). However, the time to cessation of bursting in each species was not different in the two age groups (Table 3.3; Table 3.4). Thus the age of the neonate did not affect the type of response (ie. the variable affected) or the time to ultimate failure of fictive breathing. Rather, at any given point in the experiment after the application of riluzole P0-2 rats had lower burst amplitude/area in comparison to P3-4 rats and P0-2 hamsters had lower frequency of bursting in comparison to P3-4 hamsters (Figure 3.7; Figure 3.8). These observations may reflect changes in the resting membrane potential over development. In very young neonates the resting membrane potential of respiratory-related neurons is reportedly between \(-45 \) mV and \(-50 \) mV (Richter & Spyer, 2001). Thus in very young neonates, the endogenous bursting of pacemaker cells and the network bursting of non-pacemaker cells likely depends upon a very limited set of conductances, one of which is the persistent sodium conductance. As mammals develop the resting potential becomes more negative and other conductances, such as voltage-dependent calcium, come into play and likely become involved in the generation of respiratory rhythm and pattern (Richter & Spyer, 2001). Theoretically, therefore, \( I_{\text{NaP}} \) could be relatively more important
to network bursting properties in younger rats and also relatively more important to pacemaker properties in younger hamsters.

3.5.4. Effect of cooling and re-warming on fictive breathing

Preparations from rats and hamsters responded to transitional cooling and re-warming in a similar fashion. During cooling, the frequency of fictive breathing declined in a near linear fashion and, for the most part, there was no significant effect of temperature on the pattern of the bursts. The frequency of fictive breathing prior to cooling (at 27°C) of preparations from rats and hamsters was similar in both species but somewhat different in the two age groups within each species. Since breathing frequency and metabolic rate have been found to increase during early development in both rats and hamsters in vivo it seems likely that there would be a similar increase in fictive breathing frequency with age in the in vitro preparation (Mortola, 1984, 2001). However, while P3-4 rats had higher fictive breathing frequencies than P0-2 rats, P3-4 hamsters had lower fictive breathing frequencies than P0-2 hamsters. Similar trends have been observed in other studies using in vitro preparations from rats and hamsters aged 0-4 days (Zimmer, 2002). Therefore, an increase in metabolic rate over development cannot entirely account for the differences observed in fictive breathing frequency in in vitro preparations from animals of different ages thought the trend does appear to hold true in rats.

As we observed in vivo in Chapter 2, hamster preparations typically underwent respiratory arrest at lower temperatures than rat preparations which could reflect the fact that hamsters are capable of hibernation and tolerant of very low T\textsubscript{b} whereas rats are not (Adolph, 1951; Kristofferersson & Soivio, 1966). It is worth noting that the en bloc preparations from neonatal rats and hamsters in the current study typically underwent fictive respiratory arrest at temperatures 5-10°C higher than in vivo neonates (Tattersall & Milsom, 2002; Corcoran, 2003). This difference was probably a result of decreased respiratory drive in these reduced preparations, which lack the excitatory inputs from higher centres in the brain and periphery that exist in vivo (Smith \textit{et al.}, 1990; Mellen \textit{et al.}, 2002; Zimmer, 2002). Preparations from P0-2 rats and hamsters were found to tolerate lower temperatures than preparations from older animals before undergoing respiratory arrest, the opposite finding than that of Zimmer (2002). It
appears, therefore, that while postnatal age may play a role in determining the minimum temperature tolerated by these preparations before undergoing respiratory arrest, it is likely not the only factor involved.

Cooling and re-warming en bloc preparations from rats and hamsters produced significant effects on the frequency of bursting but little change in burst amplitude, duration and area in either age group of rats and hamsters (Figure 3.9; Figure 3.10). Therefore, the primary effect of temperature was likely upon the CRG for breathing in these preparations, as it has been shown to be in other preparations (Mellen et al., 2002; Milsom et al., 2002; Tattersall & Milsom, 2003). These observations are consistent with fictive respiratory arrest having occurred at low temperatures as a result of failure of the CRG for breathing in these preparations (Mellen et al., 2002; Milsom et al., 2002). As discussed in Chapter 2, prior to ~P14 in rats and ~P30 in hamsters, neonatal mammals are capable of autoresuscitation upon re-warming from hypothermia-induced respiratory arrest. As such, all preparations of P0-2 and P3-4 rats and hamsters autoresuscitated upon re-warming from the minimum temperature. Thus the CRG of these neonatal preparations from rats and hamsters was capable of re-starting the rhythm of fictive breathing. Pacemaker properties, because they depend only upon ion availability and appropriate membrane voltage are likely to be the mechanism underlying the phenomenon of autoresuscitation. When preparations are cooled to a low temperature, endogenous bursting ceases but when they are re-warmed, endogenous bursting returns and therefore fictive breathing resumes. As discussed above, \( \text{INa_p} \) appears to have been essential for central rhythm generation in preparations from neonatal hamsters and important in network bursting properties of pattern generation in preparations from neonatal rats. The following section addresses the effects of blockade of \( \text{INa_p} \) on the ability of en bloc preparations from these species to autoresuscitate.

3.5.5. Effect of riluzole (200 \( \mu \text{M} \)) on the ability to autoresuscitate
As discussed in the previous sections, age was not an important factor in either the response to riluzole (200 \( \mu \text{M} \)) at 27°C or in the response to progressive hypothermia and subsequent re-warming from low temperatures. As such, a single age group of each species was selected for examining the role of \( \text{INa_p} \) in the phenomenon of
autoresuscitation. Riluzole did not appear to affect the ability of preparations from rats to autoresuscitate but entirely blocked autoresuscitation in 67% of preparations from hamsters (Figure 3.11; Figure 3.12). This implies that, in rats, riluzole did not alter the ability of the CRG to re-start whereas in hamsters riluzole profoundly inhibited the ability of the CRG to re-start. This is consistent with the response of preparations from rats to riluzole at 27°C, since the primary effect of the drug was not upon rhythm generation (frequency remained unaltered) and was instead upon pattern generation (burst amplitude, duration and area were significantly reduced). As discussed in Section 3.1.3, riluzole can affect the network bursting properties of non-pacemaker respiratory neurons since these properties depend on \( \text{INa}_p \) for the generation of multiple spikes comprising a single burst of activity (Denac et al., 2000; Urbani & Belluzzi, 2000; Del Negro et al., 2002b). A reduction in network bursting properties could have produced the observed declines in burst amplitude, duration and area during re-warming in the presence of riluzole. As preparations from rats were re-warmed the effect of riluzole on burst pattern appeared to increase implying that the drug had not yet exerted its full effect before re-warming took place (Figure 3.14). The low temperature may have reduced the rate at which riluzole affected these properties, in comparison to the rate of response at 27°C (Table 3.1; Figure 3.5). In contrast to rats, the majority of preparations from hamsters failed to autoresuscitate during re-warming, indicating that riluzole affected the ability of the CRG for breathing to re-start in this species. Those preparations that did autoresuscitate upon re-warming exhibited a reduction in burst amplitude and area in response to riluzole similar to the response of rat preparations. However, while riluzole appeared to affect pattern generation in these preparations from hamsters, likely via effects on network bursting properties, the primary effect of the drug was to block autoresuscitation (Figure 3.12; Figure 3.14). Thus, \( \text{INa}_p \) appears to not only be important for rhythm generation at 27°C but also for autoresuscitation from fictive respiratory arrest in preparations from hamsters.

3.6. CONCLUSIONS
In neonatal rats, riluzole did not appear to alter the generation of fictive respiratory rhythm and instead produced significant declines in burst amplitude and area, implying that riluzole blocked the \( \text{INa}_p \)-dependent network bursting properties of respiratory neurons. In addition, blockade of \( \text{INa}_p \) with riluzole failed to alter the ability of
preparations from neonatal rats to autoresuscitate from hypothermia-induced fictive respiratory arrest. The results are consistent with the hypothesis that I_{Na_p}-dependent pacemakers are not essential for rhythm generation in neonatal rats and that respiratory rhythm in this species is instead produced by a heterogenous population of pacemaker cells, some of which are not dependent on I_{Na_p}.

In contrast, blockade of I_{Na_p} with riluzole produced fictive respiratory failure in preparations from hamsters via a decline in the frequency of bursting indicating that I_{Na_p} is essential for the generation of respiratory rhythm in this species. Blockade of I_{Na_p} also blocked autoresuscitation entirely in 67% of hamster preparations. These findings are consistent with the hypothesis that I_{Na_p}-dependent pacemaker properties are essential for the generation of respiratory rhythm in neonatal hamsters. They also provide evidence that I_{Na_p}-dependent pacemaker properties of central rhythm generation are important in autoresuscitation from hypothermia-induced respiratory arrest.
4. GENERAL DISCUSSION

4.1. POSTNATAL DEVELOPMENT IN MAMMALS

As mammals develop from neonate to adult many changes occur that are of direct relevance to the development of central control of breathing. Neonatal mammals are largely unable to regulate body temperature ($T_B$) but by 15-17 days after birth (P15-17) juvenile rats and hamsters can maintain near-normal $T_B$ in a moderate cold challenge (Fairfield, 1948; Spiers & Adair, 1986; Sokoloff et al., 2000). As juveniles develop thermogenic abilities, an insulating layer of fur is grown and euthermic $T_B$ increases to that of adults and becomes less variable (Mortola, 2001; Rogalska & Caputa, 2004). Metabolic rate also increases during the first 3 weeks of life, reaching a maximum around P21 in rats and hamsters before gradually declining to adult levels (Mortola, 1984; Mortola, 2001). The respiratory reflexes, including those in response to hypoxia and hypercapnea, and the Hering-Breuer reflex are functional in the first week of life but between the second and third week these reflexes mature to produce the complex patterns exhibited by adults (Eden & Hanson, 1987; Coates & Silvis, 1999; Merazzi & Mortola, 1999; Mortola, 2001). During the first 2 weeks of postnatal development both myelination and synaptogenesis begin in the rodent brain (Davison et al. 1966; Moriizumi et al. 1995). These processes are initiated at different points in development, and occur at different rates, in the various regions of the brain (Davison et al. 1966; Moriizumi et al. 1995). All of these changes accompany the development of adult-type central control of breathing. At the start of the current experiments the overall hypothesis was that as mammals develop the central rhythm generator (CRG) for breathing matures from one that is primarily pacemaker-driven to one that is primarily network-driven, as outlined by the hybrid model of Smith et al. (2000). Thus, as the pacemaker properties of the CRG become less important to the generation of breathing over development, network properties become gradually more important.

4.2. LOSS OF PACEMAKER PROPERTIES OF RHYTHM GENERATION OVER DEVELOPMENT

One possible mechanism for the apparent loss of pacemaker properties over
development is outlined by the maturational network-burster model (Richter & Spyer, 2001). As mammals develop, the resting membrane potential of respiratory-related neurons becomes progressively more negative, bringing those cells with pacemaker properties outside the voltage range in which endogenous bursting can occur by adulthood. Endogenous bursting may still occur in the adult mammalian CRG, however, according to the maturational network-burster model, it does not occur independent of excitatory synaptic connections (Richter & Spyer, 2001). There also appears to be a developmental change in the type of pacemaker cells in the rhythm generating regions of the medulla from ones primarily dependent upon persistent sodium currents (INa_p) shortly after birth to a mixture of INa_p-dependent and calcium-dependent ones by the end of the second week of life (Pena et al., 2004). However, the active voltage range for both these sub-populations of pacemaker cells is similar indicating that the functional relevance of both groups would likely decline over development as membrane potential becomes progressively more negative (Richter & Spyer, 2001; Pena et al., 2004).

4.3. DEVELOPMENT OF NETWORK PROPERTIES OF RHYTHM GENERATION

It has been demonstrated in a number of studies that, while synaptic properties are essential to rhythm generation of breathing in adult mammals, they appear to be unnecessary for rhythm generation in neonatal mammals (Johnson et al., 1994; Ramirez & Richter, 1996; Shao & Feldman, 1997; Brockhaus & Ballanyi, 1998). During the first 2 weeks of life the role of synaptic inhibition in rhythm generation of breathing increases with the result that these mechanisms appear to be important in the generation of rhythm after P15 in rats and mice (Paton et al., 1994; Paton & Richter, 1995). As noted above, the membrane potential of respiratory neurons progressively hyperpolarises over development from neonate to adult. At some point in development, usually in the first postnatal week in rodents, the membrane potential crosses the reversal potential for chloride with the result that, in some species, γ-aminobutyric acid (GABA) is excitatory in neonates rather than inhibitory as it is in adults (Chen et al., 1996; Ballanyi et al., 1999; Ritter & Zhang, 2000; Richter & Spyer, 2001). There are also significant changes in the expression of neurotransmitters, receptors and receptor sub-types in the first 2 weeks of life of mammals. For example, it appears that the density of GABA_B receptors and glycine receptors, as well as the concentrations of GABA and glycine, in the preBötzinger complex increase over development, reaching a
peak around P12 (Liu & Wong-Riley, 2002). Not surprisingly, the developmental time scale over which this increase in inhibitory neurotransmitters occurs is correlated quite closely with the time scale over which inhibitory synaptic mechanisms become important to rhythm generation in mammals (Paton et al., 1994; Paton & Richter, 1995).

4.4. LOSS OF THE ABILITY TO AUTORESUSCITATE OVER DEVELOPMENT

Neonatal rats and hamsters are capable of autoresuscitation from hypothermia-induced respiratory arrest and suffer no apparent negative effects of either extremely low T_b or the absence of active breathing at low T_b. All neonatal rats (P0-6) and hamsters (P2-9) were found to be capable of autoresuscitation upon re-warming from very low T_b and consequently 100% of individuals of this age survived extreme hypothermia and respiratory arrest (Corcoran & Milsom, 2003; Tattersall & Milsom, 2003). These results are consistent with the hypothesis that the CRG of neonatal mammals is primarily pacemaker-driven and therefore able to re-start as long as the membrane potential is within the active range of the various conductances involved in the generation of endogenous bursting. As mammals develop, the ability to autoresuscitate is lost, perhaps as a result of shift in the relevant importance of pacemaker and network properties to rhythm generation of breathing. Juvenile rats in the current study lost the ability to autoresuscitate between 14 and 20 days of age whereas hamsters appeared to lose the ability to autoresuscitate much later, between 28 days of age and adulthood. In contrast to neonatal mammals, hypothermia-induced respiratory arrest is irreversible in adult mammals; even if the animal is artificially re-warmed, breathing fails to re-start (Adolph, 1948a). Therefore, the CRG of adult mammals must depend on processes that are permanently altered by failure of the CRG and consequently the cessation of breathing. Mechanisms such as reciprocal inhibition depend on oscillations of activity between groups of neurons (Shao & Feldman, 1997). Once the activity of one group is halted, the activity of other groups will also fail and, in order for the oscillation in activity to again occur, all of the connected groups must be re-started. The dependence of adult mammals on synaptic mechanisms of rhythm generation of breathing may be responsible for the inability of adult mammals to autoresuscitate from hypothermia-induced respiratory arrest.
4.5. ROLES OF PACEMAKER AND NETWORK PROPERTIES OF RHYTHM GENERATION IN NEONATES AND ADULTS

As discussed above, many changes occur during the development of mammals from neonate to adult. It was hypothesised at the start of the current experiments that as mammals develop there is a shift in the relevance of pacemaker and network properties of central rhythm generation of breathing. Pacemaker properties could be lost over development, through changes in membrane potential, as network properties mature into an essential element of the CRG. Thus the loss of the ability to autoresuscitate over development could occur as a result of a change in the CRG for breathing from one that is primarily pacemaker-driven in the neonate to one that is primarily network-driven in the adult. According to this hypothesis, synaptic mechanisms (network properties) of rhythm generation would not be required for breathing in neonates but would be essential for the generation of breathing in adults. Indeed, many authors have demonstrated that blockade of synaptic transmission does not affect the generation of respiratory rhythm in neonates of several species but completely blocks the generation of breathing in adults of the same species (Johnson et al., 1994; Paton et al., 1994; Paton & Richter, 1995; Ramirez & Richter, 1996; Shao & Feldman, 1997; Brockhaus & Ballanyi, 1998; Pierrefiche et al., 1998). Also according to this hypothesis, pacemaker properties of rhythm generation would be required for the generation of respiratory rhythm in neonatal mammals but not be required for the generation of breathing in adult mammals. Very little information exists on these latter two aspects of the hypothesis. The role of pacemaker properties to rhythm generation in neonatal mammals can be inferred to be an essential one simply because network properties are not required for the generation of breathing in these animals. However, there is no information, as yet, on the role of pacemaker properties in the generation of respiratory rhythm in adult mammals. In the current study the importance of pacemaker properties in the generation of respiratory rhythm in neonatal mammals was examined in two species in an attempt to provide further evidence in support of the overall hypothesis that there is a change in the mechanism of rhythm generation over development.

4.6. ROLE OF PACEMAKER PROPERTIES OF RHYTHM GENERATION IN NEONATES

As predicted by the hybrid model of central rhythm generation it appears that pacemaker properties are important, if not essential, for the generation of breathing in
neonatal mammals. Since the vast majority of pacemaker cells in the RVLM prior to P5 are dependent upon \( \text{INa}_p \) to generate endogenous bursting it was hypothesised at the start of the current study that the blockade of this current would severely perturb, if not entirely block, pacemaker properties and therefore rhythm generation (Pena et al., 2004). Interestingly, blockade of \( \text{INa}_p \) produced fictive respiratory failure via a decline in burst frequency, indicating an effect on the CRG for breathing, in en bloc preparations of hamsters but not of rats. In rats the burst frequency remained unaltered after blockade of \( \text{INa}_p \) and instead the amplitude and area of the fictive breaths declined until the breaths could no longer be distinguished. Thus, the results were consistent with the hypothesis that \( \text{INa}_p \)-dependent pacemaker properties were essential for rhythm generation in neonatal hamsters but not essential for rhythm generation in neonatal rats. Instead, it appeared that \( \text{INa}_p \) played an important role in the generation of fictive respiratory pattern (amplitude and area) in neonatal rats, perhaps through the effects of blockade of network bursting properties. Non-pacemaker respiratory neurons often fire repetitively in response to a single depolarising event and the generation of these multiple action potentials has been found to depend somewhat upon \( \text{INa}_p \) (Urbani & Belluzzi, 2000; Del Negro et al., 2002). The decline in burst amplitude and area observed in preparations from neonatal rats could indicate that the repetitive firing properties of non-pacemaker respiratory neurons were affected by the blockade of \( \text{INa}_p \) and that these properties were essential for the generation of normal respiratory pattern in these preparations. Since no significant alterations in respiratory pattern were observed in preparations from neonatal hamsters, this implies that network bursting properties in this species are produced independent of \( \text{INa}_p \) or that these repetitive firing properties are not important to the generation of fictive breathing pattern in hamsters. Perhaps the CRG of P0-4 hamsters is at an earlier stage of development than the CRG of rats of the same age and network bursting properties have yet to be developed in this species.

It was hypothesised at the start of the current experiment that the phenomenon of autoresuscitation from hypothermia-induced respiratory arrest was dependent upon the pacemaker properties of rhythm generation in neonatal mammals and that, as pacemaker properties were lost over development, the ability to autoresuscitate would also be lost. To test this hypothesis, \( \text{INa}_p \) was blocked after fictive respiratory arrest had
occurred at low temperatures, prior to re-warming. All en bloc preparations from rats autoresuscitated from fictive respiratory arrest after blockade of \( \text{INa}_p \) whereas only 33% of preparations from hamsters autoresuscitated. This implies that \( \text{INa}_p \)-dependent pacemaker properties were required for autoresuscitation in neonatal hamsters but not in neonatal rats. The results are consistent with the hypothesis that \( \text{INa}_p \)-dependent pacemaker properties are required for hamsters to autoresuscitate and that therefore the loss of these properties may be the cause of the loss of the ability to autoresuscitate over development. However, in neonatal rats \( \text{INa}_p \)-dependent pacemaker properties do not appear to be the only mechanism involved in the ability to autoresuscitate. Pena et al. (2004) reported that if both \( \text{INa}_p \)-dependent and \( \text{INa}_p \)-independent pacemaker properties (calcium-dependent) were blocked, respiratory failure occurred through a decline in the frequency of bursting. Perhaps if both subpopulations of pacemaker neurons had been blocked in the current study, rhythm generation would have failed in a similar fashion in both rats and hamsters, and that therefore autoresuscitation would not have occurred in preparations from either neonatal rats or hamsters.

4.7. SPECIES DIFFERENCES BETWEEN RATS AND HAMSTERS
Rats and hamsters live in different habitats, and have different life histories and behaviour, therefore it is not surprising that some aspects of the physiology of these two species are quite different. Hamsters are a primarily nocturnal species, live in underground burrows and typically do not aggregate unless breeding (Van Hoosier & McPherson, 1979). In contrast, rats are primarily diurnal and live above ground in loosely organised social groups (Baker et al., 1979). The most striking physiological difference between rats and hamsters that likely occurs as a result of these lifestyle differences can be found in their response to, and tolerance of, hypoxia and hypercapnea. Burrows are well established as habitats in which low \( \text{PO}_2 \) and high \( \text{PCO}_2 \) readily occur. Hamsters demonstrate low minute ventilation: oxygen consumption (\( \text{VE/VO}_2 \)), low arterial \( \text{PO}_2 \), relatively high hematocrit and a reduced sensitivity to hypoxia when compared to rats (Mortola, 1991). These physiological characteristics are often demonstrated by burrowing animals and are interpreted by many authors to be adaptations to life in a low \( \text{PO}_2/ \text{PCO}_2 \) environment. Because hamsters are more tolerant of hypoxic situations than rats, individuals of the former species would likely
survive periods of respiratory arrest (such as those that can occur in severe hypothermia) for longer than the latter species.

Rats and hamsters also inhabit environments with very different climates. Hamsters typically live in habitats with relatively cold winters and are an extremely cold tolerant species capable of hibernation (Adolph & Lawrow, 1951; Kristofferersson & Soivio, 1966; Van Hoosier & McPherson, 1979). Rats are much less tolerant of cold temperatures, inhabit areas with temperate climates and are not capable of hibernation (Adolph, 1948a; Baker et al., 1979). As such, adult hamsters survive Tb as low as 3.8°C whereas adult rats can only survive Tb as low as 15°C. Hamsters also breathe to lower Tb than rats, both in vivo (neonates and adults) and in vitro neonatal en bloc preparations (Adolph 1948b). This indicates that the CRG of hamsters, of all ages, is more tolerant of low temperatures than the CRG of rats of all ages. Since hamsters are routinely exposed to low ambient temperatures in their natural environment and voluntarily lower Tb to slightly above ambient temperatures during hibernation this difference in their CRG is not unexpected. It is important to note, however, that despite the cold tolerance of the hamster CRG, respiratory arrest occurs in severe hypothermia in this species as it does in rats and all other mammal species studied thus far. Since hibernating hamsters do not undergo respiratory arrest at very low Tb as hypothermic hamsters do it must be concluded that the states of hibernation and hypothermia are quite different in this species.

4.8. DEVELOPMENTAL DIFFERENCES IN RATS AND HAMSTERS

Both rats and hamsters are born in a very immature state, however, since hamsters are born after only 16 days gestation they may be less developed at birth than rats which are born after 21 days gestation. This difference could explain the results of the in vitro experiments described above in which blockade of INa_p produced such diverse responses in neonatal rats and hamsters. In rats the CRG could be more dependent upon network properties than the CRG of hamsters. Conversely, the CRG of hamsters appears to be dependent upon INa_p-dependent pacemaker properties of rhythm generation whereas the CRG of rats does not appear to depend exclusively upon this mechanism of rhythm generation. According to the hybrid model of central rhythm generation and the hypotheses of the current work there is a shift in relevance over
development in mammals from a primarily pacemaker-driven CRG to a primarily network-driven CRG. It may be that neonatal rats and hamsters between the ages of 0 and 4 days are at different points along the same continuum of development of an adult-type (network driven) CRG.

Neonatal hamsters are incapable of thermogenesis prior to P12 and regulate $T_b$ exclusively by behavioural mechanisms such as thermotaxis and huddling with littermates. Neonatal rats, however, are capable of non-shivering thermogenesis shortly after birth and are therefore much more effective at regulating $T_b$ than hamsters until ~P12 (Spiers & Adair, 1986; Blumberg, 1997; Sokoloff et al., 2000). After about 15 days of age rats and hamsters are both capable of shivering and non-shivering thermogenesis, have comparable locomotory skills, are similarly insulated with fur and are a similar size (relative to their adult mass). Therefore at ~P15 rats and hamsters appear to be at a similar developmental stage and indeed, juveniles of both species are typically weaned by their mothers at the same age, 21 days after birth. However, as described above, juvenile rats and hamsters lose the ability to autoresuscitate at different postnatal ages; rats between P14 and P20 and hamsters between P28 and adulthood (~P40). This difference implies that, despite the similarity in developmental time scales in these two species after ~P15, the CRG of hamsters matures at a slower rate than the CRG of rats.

4.9. OVERALL CONCLUSIONS

Over development the ability to autoresuscitate was lost in rats between the ages of 14 and 20 days after birth and in hamsters between 28 days after birth and adulthood. It was hypothesised that the loss of the ability to autoresuscitate as rats and hamsters developed corresponded to a change in the CRG for breathing from one that was primarily dependent upon pacemaker properties in neonates to one that was primarily dependent upon network properties in adults. The mechanism underlying the ability to autoresuscitate was hypothesised to depend upon the pacemaker properties of rhythm generation that predominate in neonatal mammals. Pacemaker properties were blocked using riluzole, specific for persistent sodium currents, in en bloc preparations from neonatal rats and hamsters. The results were consistent with the hypothesis that pacemaker properties were essential for rhythm generation and autoresuscitation in
neonatal hamsters but *not* essential for rhythm generation and autoresuscitation in neonatal rats. Instead, blockade of persistent sodium currents in neonatal rats appeared to affect pattern generation, perhaps through effects on network bursting properties. It may be that the different results obtained using preparations from neonatal rats and hamsters reflect developmental differences between these two species.
References


