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UMI
FUNCTIONAL CONNEXIONS AMONG
MEDULLARY RESPIRATORY NEURONES IN
THE RAT

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy, Graduate Department of Physiology, University of Toronto

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FUNCTIONAL CONNEXIONS AMONG MEDULLARY RESPIRATORY NEURONES IN THE RAT

John Howard Peever, Doctor of Philosophy
Respiratory Neuroscience Laboratory
Department of Physiology
University of Toronto

I examined the functional interconnexions among medullary respiratory rhythm-generating neurones, and the premotor pathways that transmit rhythm to hypoglossal and phrenic motoneurones using in-vivo adult, and in-vitro neonatal rat preparations.

Based on four specific research projects, the following conclusions were drawn:

1. In adult and neonatal rats, each half of the medulla contains an autonomous respiratory rhythm-generator that can function independently.

2. In adult rats, left and right hypoglossal nerve discharges are synchronised by excitation from a common premotor neurone population, as are the phrenic nerves; this type of short-term synchronisation was not detected in neonatal in-vitro preparations. I speculate that the differences between the adult and neonatal preparations are due to development of respiratory drive transmission pathways.

3. In adult rats, motoneurones of the medial and lateral hypoglossal nerve branches receive inspiratory drive from a common premotor population. In both adult and neonatal rats, hypoglossal motoneurones are not monosynaptically excited by the inspiratory neurones that are premotor to phrenic motoneurones. Hypoglossal motoneurones are not inhibited
by the Bötzinger complex expiratory neurones that inhibit phrenic motoneurones. I suggest that respiratory control of hypoglossal motoneurones is separate from that for phrenic motoneurones.

4. In adult rats, hypoglossal inspiratory motoneurones are not inhibited during expiration. Respiratory rhythm is transmitted to hypoglossal motoneurones from a distinct population of premotor neurones scattered throughout the lateral tegmental field, and perhaps by a population of interneurones within the hypoglossal motor nucleus.
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<td>XII</td>
<td>Hypoglossal</td>
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<tr>
<td>C4</td>
<td>Phrenic</td>
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<tr>
<td>NA</td>
<td>Nucleus Ambiguus</td>
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<tr>
<td>LTF</td>
<td>Lateral Tegmental Field</td>
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<td>nXII</td>
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<td>Facial Nucleus</td>
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<td>nC4</td>
<td>Phrenic Motor Nucleus</td>
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<td>nts</td>
<td>Nucleus of the Solitary Tract</td>
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<td>py</td>
<td>Pyramidal Tract</td>
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<td>sV</td>
<td>Spinal Trigeminal Nucleus</td>
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<td>VRG</td>
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<td>C2</td>
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<tr>
<td>C3</td>
<td>Third Cervical Vertebra</td>
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<td>E1</td>
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<td>Potassium Phosphate Monobasic</td>
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<tr>
<td>GABA</td>
<td>Gamma-aminobutyric Acid</td>
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<tr>
<td>aCSF</td>
<td>Artificial Cerebral Spinal Fluid</td>
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<td>μA</td>
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<td>MΩ</td>
<td>Mega-ohm</td>
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<td>M</td>
<td>Molar</td>
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<tr>
<td>µm</td>
<td>Micron or Micrometre</td>
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<tr>
<td>°C</td>
<td>Degrees Celsius</td>
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<tr>
<td>mg</td>
<td>Milligram</td>
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<td>Bursts per Minute</td>
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1.0 INTRODUCTION

The neural network responsible for the generation of respiratory rhythm is located in the brainstem. The respiratory neurones, which form the kernel of the network, are found in a bilateral column running parallel to the neuraxis. The primary function of the central respiratory system is to provide the automatic rhythm that drives the respiratory muscles, and to integrate the hierarchy of signal inputs, ranging from central chemoreceptors to pulmonary stretch-receptors, so that the system efficiently achieves its goal of O₂ and CO₂ homeostasis.

This thesis examines the brainstem respiratory network that generates and transmits respiratory rhythm to respiratory muscles in adult and neonatal rats. Specifically, I will investigate the functional interconnexions among respiratory rhythm-generating neurones, and the premotor pathways that transmit rhythm to respiratory spinal and cranial motoneurones in both in-vivo and in-vitro rat preparations.

This introductory chapter is designed to provide a general overview of our current knowledge of respiratory rhythm generation and its transmission in adult and neonatal rats. Before reviewing the neural control of breathing, a brief historical overview of the field of respiratory neurophysiology is presented.
1.1 **Historical Overview**

The earliest documented account that the brain is responsible for breathing came from the Roman physician, Galen. During the second century A.D., he recounted that gladiators injured in the neck stopped breathing, but those injured below the neck continued to breathe (Perkins, 1964). Sixteen hundred years later, Lorry showed that removing the cerebellum of rabbits did not stop their breathing, and concluded that the essential structures could be found in the brainstem and spinal cord (Feldman, 1986; Perkins, 1964). Another hundred years passed before Legallois found that a region in the brainstem, close to the exit of the vagus nerves was essential for the maintenance of breathing (Perkins, 1964). At the beginning of the twentieth century, Ramón y Cajal described three major brainstem nuclei that are important in generating breathing movements: the nucleus ambiguus, the nucleus of the solitary tract and commissural nuclei. He proposed that inherent properties of brainstem cells caused rhythmic signals to spinal and cranial motoneurones, which interacted with afferent signals from the lungs to produce breathing movements (Feldman, 1986). In 1923, Lumsden observed that the pons is an essential component of the system that produces normal breathing (eupnoea) (Lumsden, 1923). Shortly after, Adrian and Buytendijk (1931) observed that periodic oscillations in electrical potentials on the surface of the brainstem were correlated with gill movements in goldfish. In 1936, Gesell, Bricker and Magee recorded the electrical discharge of neurones in the brainstem and observed a correlation between discharge and breathing movements (Gesell et al., 1936). Since their time, “respiratory neurones”, have been recorded throughout the medulla, pons and spinal cord. Their intrinsic properties and interconnexions have been painstakingly examined in many different animals to identify the fundamental mechanisms that produce and modulate breathing movements.
In the following section, I will explain how breathing movements are accomplished and outline the basic anatomy of the brainstem respiratory network (see figure 1.0) before reviewing the detailed explanations of how rhythm is generated and transmitted to respiratory muscles.

1.2 CHARACTERISATION OF BREATHING

The primary function of breathing is to ventilate the lungs for the acquisition of oxygen and elimination of carbon dioxide. Breathing is accomplished by the rhythmic contractions of respiratory muscles, and is characterised by two phases: inspiration and expiration. Inspiration is an active process and results from contraction of thoracic muscles, which expand the chest and draw air into the lungs. Expiration is generally a passive process resulting from the elastic recoil of the lungs and thorax. While conceptually simple (air in, air out), there are many muscles located throughout the thorax and upper airways that permit effective breathing movements. For the purpose of simplicity, I will consider the main respiratory muscle, the diaphragm and one of the many upper airway muscles, the tongue. The contraction and relaxation of the diaphragm initiates airflow into and out of the lungs. While respiratory-related tongue movements do not cause air to move into the lungs they modulate the resistance to air flow. Therefore, the respiratory network in the brainstem must signal the diaphragm and tongue muscles in an appropriate sequence to achieve effective breathing and, hence lung ventilation.

The two phases of breathing, inhalation and exhalation, are controlled by three phases of neural activity: inspiration, early expiration (post-inspiration) and late expiration (see figure 1.1) (Richter, 1982; Richter et al., 1986). These neural phases are observed by
Figure 1.0. Schematic representation of the location of major respiratory groups in the rat brainstem and spinal cord. A and B are the transverse view and coronal views, respectively. Böt, Böttiger complex; DRG, dorsal respiratory group; NA, nucleus ambiguous; nVII, facial nucleus; nts, nucleus of the solitary tract; nXII, hypoglossal motor nucleus; nC4, phrenic motor nucleus; pre-Böt, pre-Böttiger complex; PRG pontine respiratory group; py, pyramidal tract; obex, anatomic reference landmark; sV spinal trigeminal nucleus; VRG, ventral respiratory group; UCIN, upper cervical inspiratory neurones.
Figure 1.1. Respiratory discharge recorded from the phrenic (A) and hypoglossal (B) nerves in a adult decerebrate rat, which illustrates the three phases of the respiratory cycle: inspiration, early expiration or post-inspiration (E1) and late expiration (E2). During inspiration both nerves discharge with the onset of the hypoglossal nerve preceding the phrenic nerve. During E2 the discharge activity of the phrenic nerve is present but declines to baseline during E2.
recording the electrical activity of respiratory muscles, such as the diaphragm, intercostal muscles and those of the upper airways, or by making extracellular recording of the nerves and motoneurones that innervate these muscles.

Although the primary function of the brainstem respiratory network is to provide the automatic rhythm that drives respiratory muscles, it receives a variety of synaptic inputs from other regions in the brain and periphery. This network must function to maximise metabolic homeostasis in the face of non-metabolic demands such as speech, chewing, swallowing, general body position, and basic reflexes like sneezing and coughing. Sensors in the periphery and within the central nervous system detect changes in partial pressures of oxygen and carbon dioxide as well as pH so that ventilation and metabolic demands are matched. The output of the respiratory network is also affected by feedback from vagal and muscle afferents, which control lung volume, rates of lung volume change, and respiratory muscle function.

1.3 Respiratory Muscle and Nerve Activity

Before considering the organisation of the central respiratory network, I will briefly categorise the major respiratory muscles and describe the discharge patterns of two major nerves innervating two major muscles, one from the thorax, and the other from the upper airways.

1.3.1 Thoracic Muscles

There are three main sets of thoracic striated muscles: the diaphragm, external and internal intercostal muscles of the rib cage, and those of the abdominal wall. Bronchial
smooth muscles serve to modulate intrathoracic airway resistance but they will not be considered further. The diaphragm and external intercostals muscles are considered inspiratory muscles because their contractions function to expand the thorax generating a sub-atmospheric intrathoracic pressure that draws air into the lungs, while internal intercostals and abdominal muscles are expiratory muscles because their contraction results in inward displacement of the rib cage and abdomen, which leads to deflation of the thorax, positive intrathoracic pressure and hence movement of air out of the lungs. Thus, thoracic muscles work in concert to produce inflation and deflations of the thorax to maintain tidal lung ventilation. Not only are these muscles used for breathing but are also required for many different behavioural acts that range from defecating to weight lifting. The intercostal muscles of the rib cage, unlike the diaphragm play a major role in maintenance of body posture and therefore their role in normal breathing is less clear than that of the diaphragm. As such, the diaphragm is considered the main respiratory muscle, and assessment of its innervation and discharge pattern during the respiratory cycle is considered next.

**Respiratory Discharge of the Phrenic Nerve:** The diaphragm is innervated by phrenic motoneurones, which are bilaterally located in two narrow columns in the medial part of the spinal cord at the level of the third to fifth cervical vertebrae (see figure 1.0). The respiratory-modulated discharge patterns of phrenic motoneurones are controlled by bulbospinal premotor neurones located in the bilateral respiratory network in the medulla.

The electrical activity of the phrenic nerve is recorded to define either the timing or the strength of respiratory activity. Figure 1.1A demonstrates a typical example of phrenic nerve activity recorded from an adult rat. The period of activity constitutes the duration of neural inspiration and the period between bursts represents neural expiration. The discharge
pattern of each phrenic burst consists of a ramping-up shape, which is due to both the increase in discharge frequency of individual phrenic motoneurones, and their progressive recruitment during inspiration (Hilaire et al., 1983; Monteau et al., 1985). Often, following the inspiratory burst of activity, the phrenic nerve discharges minimally during the early expiratory period (post-inspiratory activity). The small diaphragmatic contraction that results from this discharge may work in concert with various upper airway muscles to limit expiratory outflow, thereby making a smooth transition from inspiration to expiration (Monteau and Hilaire, 1991).

1.3.2 Muscles of the Upper Airways

Respiratory-related muscles are located at many sites of the upper airway, and can be divided into five muscle groups based on their anatomical location: those that regulate the nose, mouth, soft palate, pharynx and larynx (Iscoe, 1988). Muscles of these regions are activated mainly during inspiration to stiffen and dilate the airway conduit so that the negative pressures generated by the diaphragm and thoracic muscles does not cause the collapse of the soft tissues in the airways (Brouillette and Thach, 1980; Fregosi and Fuller, 1997; Horner, 1996; Remmers et al., 1978). For the sake of brevity and relevance to this thesis, I will discuss only the extrinsic muscles of the tongue. Tongue position is controlled primarily by the genioglossus muscle (tongue protruder), hyoglossus and styloglossus (tongue retractors) and intrinsic muscles (Iscoe, 1988; Lowe, 1981). There are functional differences between tongue retractors and protrudors (Fregosi and Fuller, 1997). Tongue protrudors maintain airway patency and have consistent inspiratory modulation (Andrew,
1955; Megirian et al., 1985; Ogawa et al., 1960), whereas the retractor muscles are inactive during normal breathing (Doty and Bosma, 1956).

Hypoglossal motoneurones innervate the muscles of the tongue and are located in bilateral nuclei extending along the midline of the brainstem at the level of, and caudal to obex (Uemura-Sumi et al., 1988; Uemura-Sumi et al., 1981). The premotor pathways that transmit respiratory drive to hypoglossal motoneurones are unknown in the rat.

Respiratory Discharge of the Hypoglossal Nerve: Because upper airway muscles control the resistance to airflow through the nasopharynx into the lungs, understanding their discharge patterns during the respiratory cycle is critical for understanding the sequence of events that characterise normal breathing patterns. While many different upper airway muscles participate in changing airway resistance, only the discharge of the hypoglossal nerve will be considered. Generally hypoglossal nerve activity precedes phrenic nerve activity (see figure 1.2B), in both decerebrate cats and anaesthetised rats; it increases activity more rapidly, and reaches an earlier peak with activity either maintained at a plateau or declining until the beginning of expiration (Fukuda and Honda, 1982a; Fukuda and Honda, 1982b; Sica et al., 1984; Sica et al., 1985). Onset times of the hypoglossal nerve when compared to those of the phrenic nerve, appear to be critically dependent on both level of anaesthesia and arterial CO₂ levels (Fukuda and Honda, 1982a; Fukuda and Honda, 1982b; Hwang et al., 1983a; Hwang et al., 1983b).

1.4 Organisation of Respiratory Centres

While the central neural mechanisms underlying the generation of respiratory rhythm are not understood, the neuroanatomical organisation of respiratory neurones is well
documented. Respiratory neurones are located in three major regions of the brainstem (figure 1.0). Respiratory neurones are classified by their discharge patterns relative to the three phases of the respiratory cycle, classically defined by phrenic nerve discharge. For example, a neurone that discharges during inspiration, is called an inspiratory neurone, whereas a neurone that fires during expiration, is called an expiratory neurone, and those that discharge during both inspiration and expiration with a maximal discharge during either phase, are termed phase-spanning neurones. However, neurones that exhibit a tonic discharge could be involved in the generation or expression of respiratory rhythm; indeed, stimulation of tonically active medullary raphé neurones modulates respiratory rhythm in both the intact animal and isolated respiratory network *in-vitro* (Bernard et al., 1996; Millhorn, 1986; Peever et al., in press). Furthermore, the condition, (i.e., anaesthetised, decerebrate, asleep, awake) of the animal preparation influences the firing pattern and spatial distribution of respiratory-modulated neurones within the brainstem rhythm-generating network (Orem and Trotter, 1992). Nevertheless, the defined medullary and pontile respiratory centres form the basic components of the central respiratory network.

The central organisation, discharge patterns, membrane properties and axonal projections of respiratory neurones have been evaluated in many mammals, including the sheep, rabbit, pig, dog, monkey and rat (Bianchi et al., 1995; Long and Duffin, 1986). However, the majority of results were obtained from adult, decerebrate or anaesthetised cats, and only recently have results come from adult or neonatal rats (Dobbins and Feldman, 1994; Ellenberger, 1999; Schwarzacher et al., 1991; Tian and Duffin, 1996b). Although the general distribution of respiratory neurones appears constant across mammalian species, recent
experiments reveal that functional connexions between some respiratory neurones differ between rat and cat (Bianchi et al., 1995; Ellenberger, 1999; Tian et al., 1998c).

1.4.1 Respiratory Neurones in the Medulla

In the medulla, respiratory neurones are concentrated in two bilateral columns that run parallel to the neuraxis. The two columns are called the ventral respiratory group and the dorsal respiratory group (figure 1.0). The dorsal respiratory group is situated within the ventrolateral division of the nucleus of the solitary tract. It contains multipolar neurones that are morphologically homogenous, which discharge primarily during inspiration. Dorsal respiratory group neurones receive primary afferent inputs from peripheral chemo- and mechanoreceptors. In cats, dorsal respiratory group bulbospinal inspiratory neurones make monosynaptic connexions with many spinal motoneurones and interneurones, including phrenic motoneurones (Aoki and Pickel, 1992; Feldman and Speck, 1983; Lipski et al., 1983b). In rats, dorsal respiratory group inspiratory neurones project to inspiratory neurones in the ventral respiratory group, but unlike the cat do not make monosynaptic connexions with spinal motoneurones, particularly phrenic motoneurones (Tian and Duffin, 1998).

The ventral respiratory group corresponds to a bilateral longitudinal column of neurones extending from the level of the first cervical spinal cord to the facial nucleus, in the region of the lateral tegmental field. Based on anatomical and functional differences, the ventral respiratory group is subdivided into the caudal, intermediate and rostral regions (figure 1.0) (Feldman, 1986; Long and Duffin, 1986).

The caudal ventral respiratory group includes part of the nucleus ambiguus and extends from the obex to the border of the medulla and spinal cord. It contains mostly
expiratory neurones that send their axons down the spinal cord but their functional connexions are unknown. Inspiratory neurones are also intermingled in the rostral portion of the caudal ventral respiratory group (Bianchi et al., 1995; Ezure, 1990).

The intermediate ventral respiratory group includes most of the nucleus ambiguus and is located in the ventrolateral brainstem at the same rostrocaudal level as the dorsal respiratory group (figure 1.0). It is synonymous with the nucleus paraambiguus (Feldman, 1986). The nucleus ambiguus contains various laryngeal motoneurones that exhibit either inspiratory or expiratory discharge patterns. The nucleus paraambiguus includes respiratory bulbospinal premotor neurones that project to spinal respiratory motoneurones, including phrenic motoneurones (Feldman, 1986; Long and Duffin, 1986). The intermediate ventral respiratory group also includes respiratory propriobulbar neurones, which Bianchi et al. (1995) suggest may coordinate the activities of thoracic pump muscles with those controlling upper airway resistance.

Located between the intermediate ventral respiratory group and the rostral ventral respiratory group is the recently termed, pre-Bötzinger complex (Feldman et al., 1990; Smith et al., 1991). In adult cats and in neonatal rats, this region is characterised by propriobulbar inspiratory, expiratory and phase-spanning neurones (Connelly et al., 1992; Schwarzacher et al., 1995; Smith et al., 1991). Some investigators hypothesise that this region contains the essential neural circuitry required to generate respiratory rhythm. This region will be discussed in more detail in sections 1.5.2 and 1.5.5.

The rostral ventral respiratory group includes the rostral portion of the nucleus ambiguus, called the retrofacial nucleus. It contains pharyngeal motoneurones with both expiratory and inspiratory discharge patterns (Ezure, 1990). It contains a population of
inhibitory, expiratory neurones called the Bötzinger complex (Merrill et al., 1983). Morphological studies of Bötzinger expiratory neurones reveal multipolar cell bodies located ventromedial to the nucleus ambiguus, with dendrites in all directions, and their main axons distribute collaterals bilaterally in the region of the nucleus ambiguus and paraambiguus nucleus (Bryant et al., 1993; Otake et al., 1987). Expiratory neurones of the Bötzinger complex exhibit an augmenting discharge pattern, and send axonal projections to the spinal cord and to neurones throughout the ventral and dorsal respiratory groups (Bianchi et al., 1988; Lipski and Merrill, 1980; Otake et al., 1987; Tian et al., 1998b), including themselves (Duffin and van Alphen, 1995b; Tian et al., 1999b). Bötzinger expiratory neurones monosynaptically inhibit phrenic motoneurones (Merrill and Fedorko, 1984; Tian et al., 1998a). Importantly, some investigators suggest that Bötzinger expiratory neurones form an essential part of the respiratory rhythm-generating network in adult mammals (Duffin et al., 1995).

1.4.2 Respiratory Neurones in the Pons

Neurones with respiratory-modulated discharge patterns are found throughout the pons but are particularly concentrated in two regions, the Kölliker-Fuse nucleus and nucleus parabrachialis medialis. These regions constitute the pontine respiratory group, also called the pneumotaxic centre (see figure 1.0) (Feldman, 1986; St.-John, 1998). Most pontine respiratory group neurones discharge tonically, with their maximal discharge occurring during inspiration, expiration, or at the transition between respiratory phases (Bianchi and St.-John, 1982; Dick et al., 1994; Vibert et al., 1976). However, the respiratory discharge of pontine respiratory group neurones is minimal when the vagal nerves are intact, but exhibit
prominent respiratory discharge when vagal nerve activity is suppressed (Feldman et al., 1976).

Pontine respiratory group neurones project to medullary respiratory neurones (Bianchi and St.-John, 1982), with a selective, perhaps polysynaptic input from the Kölliker-Fuse nucleus to the hypoglossal motor nucleus (Kuna and Remmers, 1999). It is generally agreed that pontine respiratory group neurones are not essential for the generation of respiratory rhythm, rather pontine respiratory group neurones stabilise the respiratory pattern, and influence the timing of the respiratory phases (Dick et al., 1994). However, pontine respiratory group lesions dramatically affect the expression of respiratory motor output (Jodkowski et al., 1994; Wang et al., 1993).

1.5 ANIMAL MODELS

Our understanding of respiratory rhythm-generating mechanisms is, in part, shaped by the animal preparations used to study the respiratory network. Presently, intact, in-vivo and reduced in-vitro preparations are used to address the underlying mechanisms of respiratory rhythm generation, transmission and its modulation. Each of these preparations offers particular advantages and disadvantages, which require an explanation.

1.5.1 In-vivo Preparations

In the recent past, the anaesthetised or decerebrate adult cat was exclusively used to study the brainstem respiratory network, and indeed, most of the current knowledge is derived from this preparation. However, rats have recently become the animal of choice (Bianchi et al., 1995; Monteau and Hilaire, 1991), most certainly due to their availability,
cost and size compared with the cat.

While in-vivo studies have undoubtedly pioneered and advanced our understanding of the neural control of breathing, this preparation has limitations. For example, afferent input from peripheral chemo- and mechanoreceptors are present, and both are known to alter respiratory rhythm (Feldman, 1986; Long and Duffin, 1986; St.-John, 1998). Furthermore, other central nervous structures such as the cortex and hypothalamus interact with the respiratory network in the intact animal (Waldrop and Porter, 1995). Hence, this continual modulation of the respiratory network by a multiplicity of afferent inputs complicates the analysis of the intrinsic synaptic and cellular mechanisms, which ultimately underlie the generation of respiratory rhythm in the intact animal. Accordingly, reduced preparations were developed to study the respiratory network in isolation. While several different in-vitro models have been established, there are two, which many respiratory neurophysiologists currently use: the in-vitro brainstem-spinal cord and transverse brainstem-slice from neonatal rodents.

1.5.2 In-vitro Preparations

Brainstem-Spinal Cord Preparations: In 1984, Suzue successfully isolated the central respiratory network, including both phrenic and hypoglossal motor nerve rootlets from neonatal rats (0-5 days old) (Suzue, 1984). When the brainstem-spinal cord preparation is placed into a tissue chamber perfused with artificial cerebral spinal fluid, maintained at approximately 25°C and at pH ~ 7.4, phasic bursts of activity can be recorded from phrenic and hypoglossal nerve rootlets. Suzue (1984) concluded that the spontaneously generated discharge is analogous to respiratory rhythm. Many investigators agree with this
interpretation and have accordingly used this in-vitro preparation to rigorously examine the cellular and synaptic mechanisms that generate this respiratory-like rhythm (Ballanyi et al., 1999; Hilaire and Duron, 1999; Rekling and Feldman, 1998).

**Transverse Brainstem-Slice Preparations:** In 1991, Smith, Ellenberger, Ballanyi, Richter and Feldman discovered that a thin (300-600 μm) transverse slice of the brainstem at the level of the rostral hypoglossal motor nucleus, when placed under appropriate conditions, exhibited rhythmic discharge on the hypoglossal nerve rootlets, similar to that of the brainstem-spinal cord preparation (Smith et al., 1991). They found that disrupting synaptic transmission (with low calcium concentrations) within the slice did not eliminate rhythmic fluctuations of membrane potentials in pre-Bötzing complex inspiratory neurones. From this observation was borne the hypothesis that pacemaker neurones in the pre-Bötzing complex generate respiratory rhythm, at least in this preparation.

Both experimental models offer distinct advantages for studying the central respiratory network, namely they are devoid of confounding afferent inputs, but moreover, allow a ‘systems’ approach to a reduced in-vitro preparation that can be studied at both the cellular and subcellular levels (Nattie, 1999). Also, they allow for stable electrophysiological recordings and permit pharmacological interventions due to the ease with which tissue can be manipulated, therefore permitting the analysis of synaptic and intrinsic mechanisms of respiratory rhythm generation (Ballanyi et al., 1999; Koshiya and Smith, 1999b; McCrimmon et al., 2000b; Rekling and Feldman, 1998).

Despite their utility, these techniques have disadvantages. Namely, it is unclear whether the generated rhythm in fact represents eupnoea, or an aberrant respiratory-related discharge, termed gasping (St.-John, 1998; St.-John and Paton, 2000). The frequency,
pattern and location of the hypothesised pre-Bötzinger complex rhythm-generator, have led some investigators to question the nature of the generated discharge in both *in-vitro* preparations (Ballanyi et al., 1999; Lieske et al., 2000; St.-John, 1998; St.-John and Paton, 2000). Due to this controversy, a thorough characterisation of the nature of motor and neuronal discharge patterns is in order.

### 1.5.3 Respiratory Activity of *In-vitro* Preparations

Both the brainstem-spinal cord and transverse brainstem-slice preparations exhibit similar patterns of motor output, and are therefore considered together, unless stated otherwise. Both preparations are perfused with artificial cerebral spinal fluid, which is bubbled with 95% O₂ and 5% CO₂ to supply the tissue with O₂ and to maintain pH within the physiological range ~ 7.4. The brainstem-slice preparation, unlike the brainstem-spinal cord preparation requires an unusually high concentration of potassium in the artificial cerebral spinal fluid to initiate and maintain the stereotypical motor output of hypoglossal nerves (Peever et al., 1999b; Smith et al., 1991). Both preparations are maintained at low temperatures (25-29°C) to maintain cellular viability and network function (Murakoshi et al., 1985; Smith et al., 1990; Suez, 1984). Under these conditions, the phasic motor output recorded from either the phrenic or hypoglossal nerves occurs at approximately 5-15 bursts/min (see figures 1.2A and 1.3A), with the individual bursts consisting of rapidly-peaking and slowly-decrementing discharge patterns (Ballanyi et al., 1999; Peever et al., 1999b).

In preparations where the brainstem-spinal cord remain attached to the dorsal part of
Figure 1.2. The effect of increasing artificial cerebral spinal fluid temperature on the bursting frequency and pattern of hypoglossal (XII) nerve discharge recorded in an in-vitro brainstem-slice preparation made from a neonatal rat. A: a graphical representation of how temperature affects XII bursting frequency; note, at low temperatures XII bursting frequency is similar to the frequency of gasping recorded from an intact neonatal rat (Wang et al. (1996)). B: raw (top traces) and integrated (bottom traces) records of XII nerve discharge at both 25°C and 35°C. (Modified from: Peever et al. 1999b)
A: Effect of Temperature on Phrenic Burst Frequency

- Eupnoea-Range *In-vivo*
- Gasping-Range *In-vivo*

B: Effect of Temperature on Phrenic Burst Pattern

Figure 1.3. The effect of increasing artificial cerebral spinal fluid temperature on the bursting frequency and pattern of phrenic nerve discharge recorded in an *in-vitro* brainstem-spinal cord preparation made from a neonatal rat. A: a graphical representation of how temperature affects phrenic bursting frequency; note, at low temperatures phrenic bursting frequency is similar to the frequency of gasping recorded from an intact neonatal rat (Wang et al. 1996). B: raw (top traces) and integrated (bottom traces) records of phrenic nerve discharge at both 25°C and 35°C. (Modified from: Peever et al. 1999b)
the thorax, spontaneous upward movements of the thorax occur synchronously with phasic bursts of phrenic nerve rootlet activity. In addition, it was also demonstrated that the phrenic nerve discharged in phase with the inspiratory external intercostals muscles (Smith et al., 1990; Suzue, 1984). Also, in brainstem-spinal cord preparations, in which the lungs and vagal nerves are intact, either lung inflation or vagal nerve stimulation inhibited phasic bursts (Mellen and Feldman, 1997; Mellen and Feldman, 2000; Murakoshi and Otsuka, 1985).

Both in-vitro brainstem-spinal cord and transverse brainstem-slice preparations respond to altered CO₂/pH. An increase in the concentration of CO₂ (5 % to 10 %) in the artificial cerebral spinal fluid sufficient to decrease pH from ~7.4 to 7.0-6.5 caused the frequency of phasic bursts to increase from 5-15 bursts/min to 25-30 bursts/min, however, burst amplitude was generally unaffected (Johnson et al., 1997; Kawai et al., 1996; McLean and Remmers, 1994; Murakoshi et al., 1985; Peever et al., 1999b; Suzue, 1984).

1.5.4 Interpretation of Discharge of In-vitro Preparations

In in-vitro brainstem-spinal cord and transverse brainstem-slice preparations the frequency of phasic bursts range from 5-15 bursts/min, with individual bursts exhibiting a rapidly-peaking then slowly-decrementing discharge pattern. This slow and aberrant discharge pattern closely resembles the ‘gasping-like’ pattern that can be induced in intact animals by transecting the brainstem at the level of the pons and medulla, or by exposure to severely hypoxic or anoxic conditions (Lieske et al., 2000; St.-John, 1998; St.-John and Paton, 2000; Telgkamp and Ramirez, 1999). It is argued that bursting frequency of these preparations is slow because the temperature is low, and that cutting vagal afferents not only slows bursting frequency but also initiates the rapidly-peaking slowly-decrementing
discharge patterns of individual phasic bursts (Smith et al., 1990). Indeed, when the temperature is increased to within the physiological range (35°C), the frequency and duration and shape of phasic bursts approach that of intact, age-matched neonatal rats (see figures 1.2B and 1.3B) (Peever et al., 1999b; Smith et al., 1990). Similarly, removal of vagal afferents in anaesthetised intact neonatal rats, slowed inspiratory bursting and transformed the inspiratory bursts from the typical ramping-up pattern to the rapidly-peaking slowly-decrementing pattern of the in-vitro preparations (Smith et al., 1990). These observations are consistent with the argument that the ‘gasping-like’ pattern of in-vitro preparations results from low temperature and lack of vagal input.

However, Wang et al. (1996) observed that the typical ramping-up pattern of eupnoea at its characteristic frequency (~35-45 bursts/min) was quickly changed to a gasping pattern, that is, rapidly-peaking, slowly-decrementing discharge patterns and slower frequencies (~10-15 bursts/min) when vagotomized, anaesthetised or decerebrate neonatal rats were made hypoxic. Furthermore, the eupnoeic discharge pattern was not converted to a rapidly-peaking, slowly-decrementing gasping pattern following vagotomy unless the animals become concomitantly hypoxic. More recently, St.-John and Paton (2000) have demonstrated that when the arterially perfused in-situ brainstem is made hypoxic, the eupnoeic-like pattern of phrenic nerve activity is transformed into a gasping-like pattern (Paton, 1996). This is consistent with the arguments that in-vitro preparations indeed generate a gasping-like pattern due to the hypoxic conditions of the tissue.

In brainstem-spinal cord preparations, Okada et al. (1993) measured the depth profiles of extracellular pH and partial pressure of O2 and reported that tissue in the vicinity of the ventral respiratory group is severely hypoxic and hypercapnic. They conclude that
very superficial regions of the tissue must be important in the generation of the respiratory-like discharge. However, Brockhaus et al. (1993) found that baseline levels and activity-related changes of extracellular potassium, calcium and pH in the region of the ventral respiratory group are stable for hours, and indeed comparable to those observed in intact rats. They suggest that ion homeostasis in the ventral respiratory group region is not pathologically disturbed due to hypoxia, and it is therefore unlikely that in-vitro brainstem-spinal cord preparations generate a gasping-like pattern due to anoxic conditions.

Also, bathing either brainstem-spinal cord or transverse brainstem slice preparations with hypoxic artificial cerebral spinal fluid results in a typical bi-phasic response, that is, an initial augmentation in the frequency of rhythmic discharge followed by a progressive slowing of discharge; this is the characteristic response to hypoxemia in intact neonatal mammals, including rats (Lieske et al., 2000; Rigatto, 1984; Telgkamp and Ramirez, 1999). While both in-vitro preparations respond to decreased pH by increasing the frequency of bursting discharge, this response is unlike that in-vivo, in which the amplitude of bursts increases (Peever et al., 1999b), but bursting frequency is unaltered (Zhou et al., 1996). If the generated discharge were gasping, bursting frequency should not respond to pH/CO₂, because gasping is insensitive to hypercapnia in the intact cat (St.-John and Knuth, 1981). Although in-vitro and in-vivo preparations respond differently to altered CO₂/pH, this difference may reflect a temperature dependence of the CO₂/pH response (Peever et al., 1999b).

Recently, Lieske et al. (2000) found that both gasping and eupnoeic discharge patterns could be generated in in-vitro transverse brainstem slices; during normal oxygenation an eupnoea discharge pattern was recorded, but under anoxic conditions a
gasping pattern emerged. They conclude that a single medullary network is conditionally reconfigured to generate both eupnoeic and gasping discharge patterns. It is therefore possible that the brainstem-spinal cord and transverse brainstem-slice preparations contain the appropriate neural circuitry to generate either eupnoea or gasping, and factors such as tissue oxygenation, pH, and temperature, ultimately determine the pattern of motor discharge (Lieske et al., 2000; Peever et al., 1999b; St Jacques and St.-John, 2000; St.-John and Paton, 2000).

1.5.5 Respiratory Neurones of In-vitro Preparations

Inspiratory, expiratory and phase-spanning neurones are located between 50-500 μm below the ventral surface of the medulla in a bilateral column extending from the caudal region of the facial nucleus to the pyramidal decussation, in a region roughly analogous to the nucleus ambiguus or ventral respiratory group in adult animals (Arata et al., 1990; Hilaire et al., 1990; Onimaru et al., 1987; Smith et al., 1990). The presence of a region comparable to the dorsal respiratory group is controversial; Hilaire et al. (1990) found no evidence of respiratory neurones in the dorsal respiratory group region in brainstem-spinal cord preparations. However, in a recent abstract, Wilson et al. (1999) report that in transverse brainstem slices, inspiratory neurones are located in a region approximating the dorsal respiratory group of adult rats.

While the intrinsic membrane potentials of inspiratory and pre-inspiratory neurones, putative pacemaker neurones, have been extensively reported, virtually nothing is known about either the axonal projections of these respiratory neurones or the functional interconnexions among them. However, two studies using cross-correlation analysis
demonstrated that pre-inspiratory and inspiratory neurones in the ventral respiratory group/pre-Bötzinger complex make excitatory connexions with one another (Kashiwagi et al., 1993; Onimaru et al., 1993). How respiratory drive is transmitted to either hypoglossal or phrenic motoneurones is unknown in these preparations. To determine the mechanisms of respiratory rhythm generation and its transmission, a detailed understanding of the synaptic connexions among rhythm-generating neurones and their premotor pathways is required.

Recently, Koshiya and Smith (1999b) used an activity-dependent fluorescent dye to visualise respiratory neurones that send axons across the midline of the transverse brainstem slice from neonatal rats. They report inspiratory neurones in the region of the pre-Bötzinger complex discharge in phase with the rhythmic discharge of hypoglossal nerve rootlets, and also demonstrate that these cells continue to rhythmically discharge after synaptic transmission is blocked. They conclude that pacemaker neurones generate the respiratory-like rhythm, and form the ‘kernel’ or ‘noeuud vital’ of the respiratory rhythm-generator.

1.6 The Respiratory Rhythm Generator

Although the central location of respiratory neurones is well documented (i.e., ventral respiratory group, dorsal respiratory group and pontine respiratory group), the pathways and mechanisms that ultimately generate respiratory rhythm are unclear. To determine the role that different parts of the respiratory network play in rhythm generation, specific regions of interest have been lesioned or stimulated. While such an approach has limitations, it is a useful starting point for the identification of the key components of the respiratory rhythm-generator.
Many investigators have demonstrated that the pons is essential in the expression of eupnoea in both adult cats and rats (Bertrand and Hugelin, 1971; Jodkowski et al., 1994; Lumsden, 1923; St.-John and Knuth, 1981; Tang, 1967). Removal of the pons or lesions of the pontine respiratory group transform eupnoea to either apneusis or gasping, depending on the lesion site (Lumsden, 1923). Nonetheless, such experiments clearly illustrate that the pons forms an integral part of essential neural circuitry required for the expression of normal respiratory rhythm.

Unlike the ventral respiratory group, the dorsal respiratory group does not appear essential in respiratory rhythm generation. Berger and Cooney (1982) found that injection of the neurotoxin, kainic acid into the dorsal respiratory group of anaesthetised adult cats caused a decrease in the frequency of respiratory rhythm, but did not eliminate it; even after several weeks recovery, awake cats continued to breathe normally, albeit at slower frequencies.

However, lesions in the ventral respiratory group region both in-vivo and in-vitro have profound effects on the expression of respiratory rhythm. In rabbit, cat, and rat an area in the vicinity of the retrofacial nucleus appears important in the expression of respiratory rhythm. Flourens was the first to describe a “noeud vital” or ‘essential region’ in the medulla around the level of the obex, which when destroyed eliminated respiratory rhythm of the anaesthetised cat (von Euler, 1986). Focal cooling or injection of the sodium channel blocker, procaine into the retrofacial nucleus abolished respiratory rhythm in anaesthetised rabbits (Budzinska et al., 1985; McCrimmon et al., 2000a; Zhang et al., 1991). Bilateral lesions of the retrofacial nuclei with kainic acid abolished phasic phrenic activity in decerebrate cats (St.-John et al., 1989). Unilateral microinjections of a glutamate antagonist into the retrofacial nucleus resulted in complete cessation of respiratory rhythm in
anaesthetised rats (Jung et al., 1991). Koshiya et al. (1993) found that the GABA agonist, muscimol when bilaterally injected close to the retrofacial nuclei abolished rhythm in the anaesthetised rat. Similarly, in cats, the presynaptic calcium-channel blocker, ω-conotoxin GVIA was used to block synaptic transmission unilaterally in the pre-Bötzingger complex (retrofacial nucleus) and induced transient apnoea. Injection of the sodium-channel blocker, tetrodotoxin irreversibly abolished eupnoea, however, gasping could be induced when cats were made hypoxic or asphyxic (Ramirez et al., 1998).

These observations are consistent with the well-established hypothesis that the respiratory rhythm-generator resides specifically in the rostral part of the ventral respiratory group (Ballanyi et al., 1999; Bianchi et al., 1995; Duffin et al., 1995; Ezure, 1990; Feldman, 1986; Hilaire and Duron, 1999; Long and Duffin, 1986). Moreover, some investigators postulate that the rhythm-generator resides in the region of the retrofacial nucleus or pre-Bötzingger complex (Ballanyi et al., 1999; Koshiya and Smith, 1999b; Lieske et al., 2000; McCrimmon et al., 2000b; Rekling and Feldman, 1998). Such a view is reinforced by the observation that the pre-Bötzingger complex can be isolated in the in-vitro transverse brainstem-slice preparation, which continues to exhibit a spontaneous respiratory-like rhythm on hypoglossal nerve rootlets (Gray et al., 1999; Koshiya and Smith, 1999b; Ramirez et al., 1996; Smith et al., 1991).

1.6.1 Bilateral Interactions

A bilateral distribution of the respiratory network is consistent with the bilateral organisation of respiratory motoneurones and muscles. Whether each half of the bilateral ventral respiratory group network is capable of generating respiratory rhythm independently
is controversial. In adult rabbits and monkeys, left and right phrenic nerve activities become asynchronous after a mid-sagittal section of the brainstem (Gromysz and Karczewski, 1982; Janczewski and Karczewski, 1984; Karczewski and Gromysz, 1982). However, in the cat, this same procedure abolishes bilateral phrenic nerve activity (Gromysz and Karczewski, 1982; Kubin et al., 1987; St.-John, 1983), as it does in in-vitro brainstem-spinal cord preparations (McLean and Remmers, 1994). The latter observations are consistent with unilateral lesions studies, which reveal that focal destruction of the retrofacial nucleus abolishes the expression of respiratory rhythm (Jung et al., 1991; Onimaru et al., 1988; Ramirez et al., 1998). Together these studies demonstrate that in neonatal rats and adult cats both sides of the medulla are required for the expression of respiratory rhythm. It is undetermined whether both halves of the medulla are required to express respiratory rhythm in adult rats. However, in a recent abstract Janczewski and Aoki (1997) demonstrate that midline transection of the medulla abolishes respiratory rhythm in anaesthetised neonatal rats (2-9 days old), but in older rats (17-21 days old) it does not. From this preliminary observation, it might be suggested that the bilateral organisation of the respiratory network is subject to developmental change.

1.6.2 Developmental Aspects

The respiratory rhythm-generating network is subject to developmental change, but the underlying processes are poorly understood, and are generally attributed to immature cellular and sub-cellular properties (Ballanyi et al., 1999; Hilaire and Duron, 1999). However, developing network connexions might, in part, explain developmental changes in network output. Indeed, it has been reported that the strength of coupling between pre-
Bötzingen complex neurones and hypoglossal motoneurones changes during postnatal development. In transverse brainstem-slice preparations from neonatal mice, Ramirez et al. (1996) demonstrate that in 0-9 day-old mice, pre-Bötzingen complex inspiratory neurones and the hypoglossal nerve discharge in a 1:1 ratio, however, at older ages (> 9 day-old), this ratio becomes 3:1. In neonatal rats unlike adults, gap junctions electrically couple hypoglossal motoneurones to one another, like motoneurones in the nucleus ambiguus (Mazza et al., 1992; Rekling and Feldman, 1997). It is assumed that electrically coupled motoneurones form a syncytium so that developing synaptic inputs are distributed homogeneously throughout the motoneurone population.

The firing properties of putative rhythm-generating neurones change during the course of development. In in-vitro preparations, inspiratory and pre-inspiratory neurones exhibit a decrementing discharge pattern during neural inspiration (Onimaru et al., 1988; Smith et al., 1991; Smith et al., 1990), but in mature animals in-vivo, their discharge pattern is augmenting (Connelly et al., 1992; Schwarzacher et al., 1995). Also, in in-vitro preparations from neonatal mice, the shape of hypoglossal nerve discharge changes from a rapidly-peaking, slowly-decrementing pattern to a bell-shaped pattern during the first few weeks of postnatal development (Ramirez et al., 1996). Moreover, it is well established that the role of GABAergic and glycinergic inhibition changes profoundly during early development (Lieske et al., 2000; Paton and Richter, 1995a; Paton and Richter, 1995b). Thus, it is hypothesised that developing inhibitory connexions among rhythm-generating neurones and excitatory connexions to motoneurones change during development thereby altering neural discharge patterns and motor output expression (Ballanyi et al., 1999; Hayashi and Lipski, 1992; Paton and Richter, 1995b; Rekling and Feldman, 1998). Therefore,
characterising functional connexions among respiratory neurones is a prerequisite to understanding the mechanisms of rhythm-generation, and how rhythm is transmitted to and shapes the discharge pattern of respiratory motoneurones.

1.7 Respiratory Rhythm Generation

The cellular and network processes that generate respiratory rhythm are unknown. To explain the process of rhythm-generation, three models have been established, the network, pacemaker and hybrid models. These models rely on fundamentally different mechanisms for the generation of rhythmic respiratory output, but, all require tonic excitatory inputs to generate oscillatory behaviour.

The network model predicts that mutual inhibition between populations of inhibitory respiratory neurones form an oscillator, which underlies respiratory rhythm generation. Two-phase and three-phase oscillating networks have been modelled (Duffin, 1991; Ezure, 1990; Richter et al., 1992); both use the firing patterns of electrophysiologically identified respiratory neurones and their synaptic connexions to predict network behaviour. In a two-phase model, ventral respiratory group decrementing inspiratory and expiratory neurones form the core of the rhythm-generator, with tonic excitatory inputs, such as those from central chemoreceptors maintaining appropriate levels of neuronal excitation (Duffin et al., 1995; Cohen, 1995). Computer simulations show that a mutual inhibition between decrementing inspiratory and expiratory neurones forms a bi-stable oscillator, capable of generating a sustained rhythm (Duffin, 1991).

Because the network model predicts that synaptic inhibition is required to generate respiratory rhythm, blockade of inhibitory processes should therefore disrupt it; in adult rats,
this is indeed the case. Hayashi and Lipski (1992) found that drugs affecting GABAergic and glycinergeric transmission or perfusion of the arterially perfused in-situ brainstem-spinal cord of adult adults with low concentrations of chloride, severely disrupted respiratory rhythm. They concluded that synaptic inhibition is required for the generation of respiratory rhythm in the adult respiratory network.

The pacemaker hypothesis predicts that intrinsic membrane properties of respiratory neurones or groups of neurones produce rhythmic oscillations in membrane potentials, which form the core of a rhythm generator (Butera et al., 1999; Koshiya and Smith, 1999a; Rekling and Feldman, 1998). This model does not rely on inhibitory connexions for rhythm generation. Indeed, in in-vitro brainstem-spinal cord and transverse brainstem-slice preparations, blocking GABAergic and glycinergeric transmission or reducing extracellular chloride to zero does not eliminate respiratory-like rhythm (Onimaru et al., 1990; Ramirez et al., 1996; Shao and Feldman, 1997). Based on this observation, some investigators suggest that inhibitory processes are not required to generate respiratory rhythm. Instead, they hypothesise that respiratory rhythm is generated by the intrinsic pacemaker properties of pre-Bötziungen complex inspiratory neurones (Ballanyi et al., 1999; Gray et al., 1999; Koshiya and Smith, 1999b; Lieske et al., 2000; McCrimmon et al., 2000b; Rekling and Feldman, 1998). The discrepancy between observations in-vivo and in-vitro led to the suggestion that respiratory rhythm is produced by a hybrid-pacemaker-network, with pacemaker processes dominating in the neonate and inhibitory network processes dominating in the adult (Duffin et al., 1995).
1.8 Respiratory Rhythm Transmission

Respiratory rhythm must be transmitted to muscles of the upper airways and diaphragm in an appropriate sequence to allow for efficient lung ventilation. It is therefore essential to understand how the respiratory rhythm-generating network transmits drive to respiratory motoneurones. Respiratory drive is transmitted to spinal respiratory motoneurones by bulbospinal neurones concentrated in the ventral respiratory group and dorsal respiratory group (Duffin et al., 2000). Inspiratory bulbospinal premotor neurones are located in both the ventral respiratory group and dorsal respiratory group, whereas, expiratory bulbospinal respiratory premotor neurones are located in the caudal ventral respiratory group and Bötzinger complex of the rostral ventral respiratory group. The respiratory neurones that transmit drive to motoneurones innervating upper airway muscles are presumed to arise from the same regions as those for spinal motoneurones; however, little is known about the pathways of rhythm transmission to these motoneurones.

As previously noted, many muscles of the thorax and upper airways are involved in breathing. However, to remain consistent with the thesis topic, only the motoneurones innervating the tongue and diaphragm will be addressed.

1.8.1 Respiratory Drive to Phrenic Motoneurones

In both rats and cats, intracellular recording from phrenic motoneurones reveal that they depolarise during inspiration and repolarize during expiration (Berger, 1979; Cameron et al., 1991b; Gill and Kuno, 1963; Hayashi and Fukuda, 1995; Tian et al., 1998c). In cats, phrenic motoneurone membrane potential trajectories show only 2 stages, inspiratory and expiratory; an abrupt repolarization at the onset of expiration followed by a slow
hyperpolarization during expiration, which reaches a maximum at the end of expiration (Berger, 1979; Merrill and Fedorko, 1984). However, in decerebrate rats, phrenic motoneurones exhibit a 3-stage pattern of membrane potential trajectory, which is characterised by depolarisation during inspiration with a 2-stage hyperpolarization during expiration; an abrupt repolarisation then slow depolarisation in early expiration, followed by a further hyperpolarization in late expiration (Tian et al., 1998c). The membrane potential trajectories of phrenic motoneurones in in-vitro brainstem-spinal cord preparations of neonatal rats are quite different; during inspiration the membrane potential declines after its initial rapid rise, and during expiration the rapid repolarisation at the onset of expiration is not obvious (Liu et al., 1990; Parkis et al., 1999).

The respiratory-related membrane potential trajectories of phrenic motoneurones are governed by inspiratory and expiratory bulbospinal neurones in the ventral respiratory group and dorsal respiratory group, which have extensive axonal arborisations within the phrenic motor nucleus (Dobbins and Feldman, 1994). In cats, respiratory drive is monosynaptically transmitted to phrenic motoneurones primarily by dorsal respiratory group bulbospinal inspiratory neurones. Cohen et al. (1974) cross-correlated the inspiratory activities of dorsal respiratory group neurones and the phrenic nerve, and reported that ~ 70 % of the cross-correlation histograms revealed positive features. Typically, cross-correlation histograms computed with the contralateral phrenic nerve exhibited narrow peaks at short latencies, indicative of monosynaptic excitation. Similarly, in cats, Lipski et al. (1983a) used spike-triggered averaging to demonstrate that contralateral bulbospinal dorsal respiratory group inspiratory neurones project to and elicit excitatory postsynaptic potentials in phrenic motoneurones. Using the same approach, Fedorko et al. (1983) found excitatory
postsynaptic potentials occurred in 31 of 47 pairs of contralateral dorsal respiratory group inspiratory neurones and phrenic motoneurones.

In rats, very few dorsal respiratory group neurones appear to functionally project to phrenic motoneurones. De Castro et al. (1994) found that only ~ 12 % of dorsal respiratory group inspiratory neurones could be antidromically activated from the third cervical segment of the spinal cord. Similarly, Tian and Duffin (1998) found that only 5.3 % of dorsal respiratory group inspiratory neurones could be antidromically activated from the seventh cervical segment of the spinal cord in decerebrate rats. They also found that cross-correlation histograms computed between the inspiratory activities of dorsal respiratory group neurones and left and right phrenic nerves, revealed histograms with central peaks, but there was no evidence for monosynaptic connexions with phrenic motoneurones.

In cats, few bulbospinal ventral respiratory group inspiratory neurones monosynaptically excite phrenic motoneurones compared with bulbospinal dorsal respiratory group inspiratory neurones. Cross-correlation histograms computed between the inspiratory activities of ventral respiratory group neurones and the phrenic nerve reveal that 27-59 % of ventral respiratory group project to and monosynaptically excite phrenic motoneurones (Fedorko et al., 1983; Feldman and Speck, 1983). However, studies that used spike-triggered averaging between extracellularly recorded ventral respiratory group inspiratory neurones and intracellular phrenic motoneurone membrane potentials found few (2-30 %) examples of excitatory postsynaptic potentials (Fedorko et al., 1989; Fedorko et al., 1983). Nevertheless, it is generally concluded that few ventral respiratory group inspiratory neurones project to and monosynaptically excite phrenic motoneurones (Monteau and Hilaire, 1991).

From neuroanatomical experiments in cats, it was observed that ventral respiratory
group bulbospinal neurones send axons that project to the soma and dendrites of labelled phrenic motoneurones; no axon terminals were located outside the phrenic motor nucleus (Ellenberger and Feldman, 1988; Feldman et al., 1985). These investigators concluded that ventral respiratory group bulbospinal neurones project to and monosynaptically excite phrenic motoneurones, and suggested that spinal interneurones do not transmit drive to phrenic motoneurones. In rats, Dobbins and Feldman (1994) found that the transsynaptic transport of pseudorabies virus from the phrenic nerves labelled phrenic motoneurones, and ventral respiratory group bulbospinal neurones, but almost no dorsal respiratory group neurones were labelled. In agreement with this observation, two studies in rats, which used cross-correlation analysis, report that almost all bulbospinal ventral respiratory group inspiratory neurones via bilateral connexions project to and monosynaptically excite phrenic motoneurones (Duffin et al., 2000; Duffin and van Alphen, 1995a; Tian and Duffin, 1996b).

To observe the pattern of synaptic inhibition in phrenic motoneurones, chloride iontophoresis has been used to reverse inhibitory hyperpolarization to depolarisation. In both rats and cats, hyperpolarization during expiration was reversed to a depolarisation, which indicates that phrenic motoneurones are actively inhibited during the expiratory period (Berger, 1979; Hayashi and Fukuda, 1995; Tian et al., 1998c). Because expiratory neurones of the caudal ventral respiratory group do not have axonal arborisations within the phrenic motor nucleus, it is unlikely that they actively inhibit phrenic motoneurones. Indeed, Fedorko and Merrill (1984) found no evidence for functional projections from caudal ventral respiratory group expiratory neurones and phrenic motoneurones using spike-triggered averaging in adult cats. However, in both rats and cats, expiratory neurones of the Bőtzinger complex send axons to the region of the phrenic motor nucleus (Dobbins and Feldman, 1994;
Fedorko and Merrill, 1984; Núñez-Abades et al., 1991; Otake et al., 1987). In both rat and cat, spike-triggered averaging was used to demonstrate the existence of inhibitory postsynaptic potentials produced in phrenic motoneurones by Bötzinger expiratory neurones (Duffin et al., 2000; Merrill and Fedorko, 1984; Tian et al., 1999a). This method revealed individual inhibitory postsynaptic potentials in phrenic motoneurones that were correlated with the action potentials of bilaterally located Bötzinger expiratory neurones, which indicates that these neurones monosynaptically project to and inhibit phrenic motoneurones.

1.8.2 Respiratory Drive to Hypoglossal Motoneurones

Despite the significance of hypoglossal motoneurones in maintaining airway patency during inspiration and their supposed role in obstructive sleep apnoea (Harper and Sauerland, 1978; Horner, 1996; Remmers et al., 1978), relatively little is known about how respiratory rhythm is transmitted to them. Like phrenic motoneurones, intracellular recordings from hypoglossal motoneurones in adult cats reveal fluctuations in membrane potential with the respiratory cycle; during inspiration, they depolarise and during expiration repolarise (Withington-Wray et al., 1988; Woch and Kubin, 1995). Injecting chloride current into hypoglossal motoneurones to reverse inhibitory hyperpolarization to depolarisation has no effect on membrane potential trajectories, which demonstrates that hypoglossal motoneurones are not actively inhibited during expiration (Withington-Wray et al., 1988; Woch and Kubin, 1995).

Numerous neuroanatomical studies in both cats and rats identify hypoglossal premotor neurones throughout the medulla and pons (Borke et al., 1983; Dobbins and Feldman, 1995; Ono et al., 1994; Ugolini, 1995). Using transsynaptic transport of
pseudorabies virus from the medial and lateral branches of the hypoglossal nerve in rats, Dobbins and Feldman (1995) identified first-order hypoglossal premotor neurones in the lateral tegmental field. In cats, Ono et al. (1994, 1998) demonstrated that inspiratory neurones in the region of the ventral respiratory group (nucleus ambiguus) and lateral tegmental field project to and excite both hypoglossal and phrenic motoneurones. In rats, the source of respiratory drive to hypoglossal motoneurones is unknown.

1.9 Summary

In adult rats, a bilateral network of respiratory neurones located within the brainstem generates respiratory rhythm. Respiratory rhythm is monosynaptically transmitted to phrenic motoneurones by a bilateral network of excitatory and inhibitory bulbospinal premotor neurones located in the rostral ventral respiratory group. The premotor circuitry that transmits respiratory drive to hypoglossal motoneurones is unknown.

The isolated respiratory network contained in the in-vitro brainstem-spinal cord and transverse brainstem-slice preparation when placed into a well oxygenated artificial cerebral spinal fluid at an appropriate temperature and pH generates rhythmic bursts of activity on phrenic and/or hypoglossal nerve rootlets. It is generally assumed that this phasic discharge represents respiratory rhythm; however, some investigators believe it represents gasping. The bilateral organisation of the medullary respiratory-rhythm generator, and the premotor circuitry that transmits respiratory drive to phrenic and hypoglossal motoneurones in the in-vitro preparations is largely unknown.
1.10 Objectives

The general aim of this doctoral thesis is to examine the functional interconnexions among the medullary respiratory neurones and hypoglossal and phrenic motoneurones in both in-vivo adult and in-vitro neonatal respiratory networks. My specific research objectives are listed below:

Objective 1 (Chapter 3): To determine whether each side of the medulla contains an autonomous respiratory rhythm-generator in both in-vivo adult, and in-vitro neonatal rat preparations.

Objective 2 (Chapter 4): To examine the “short-term” synchronisation of bilateral (left and right) hypoglossal and phrenic nerve activities in both in-vivo adult, and in-vitro neonatal rat preparations.

Objective 3 (Chapter 5): To determine whether hypoglossal and phrenic nerves receive respiratory drive from a common premotor population in both in-vivo adult rats and in-vitro brainstem-spinal cord preparations.

Objective 4 (Chapter 6): To determine how respiratory rhythm is transmitted to hypoglossal motoneurones in adult rats.
CHAPTER 2

EXPERIMENTAL METHODS

2.0 GENERAL CONSIDERATIONS

To examine the intact medullary respiratory network I used the *in-vivo* unanaesthetised, decerebrate adult rat preparation, and to examine the isolated neonatal network, I used the *in-vitro* brainstem-spinal cord and transverse brainstem slice preparations from 1-5 day-old neonatal rats. The surgical and procedural methods used for their manufacture are outlined in section 2.1. All procedures were reviewed and approved by the University of Toronto animal care committee.

To achieve the objectives outlined in Chapter 1, section 1.10, a variety of experimental techniques were used. To clarify and put into context the methods and procedures described in this chapter, I will briefly list the technical approaches used in each of the four projects outlined in Chapters 3 to 6.

**Chapter 3:** A scalpel blade was used to *surgically lesion* the midline of the medulla while the respiratory activity of the left and right phrenic nerves was monitored in adult decerebrate rats *in-vivo*. In *in-vitro* brainstem-spinal cord and transverse brainstem-slice preparations, mid-sagittal *lesions* of the spinal cord and medulla were made while respiratory-like rhythm was monitored.

**Chapter 4:** In both *in-vivo* and *in-vitro* brainstem-spinal cord and transverse brainstem-slice preparations, the short-term synchronisation of the inspiratory activities of
left and right hypoglossal nerves, and left and right phrenic nerves were assessed using *cross-correlation analysis*.

**Chapter 5:** In both *in-vivo* and *in-vitro* brainstem-spinal cord preparations, the short-term synchronisation between the inspiratory activities of hypoglossal and phrenic nerves, and between adjacent branches of the hypoglossal nerve were assessed with *cross-correlation analysis*. In *in-vivo* preparations, the *extracellular* activity of ventral respiratory group inspiratory neurones was *cross-correlated* with the inspiratory activities of both hypoglossal and phrenic nerves.

**Chapter 6:** In the *in-vivo* adult rat preparation, membrane potentials of hypoglossal motoneurones were *intracellularly* recorded, and *chloride iontophoresis* used to reverse hyperpolarization to depolarisation. The inspiratory activity of neurones located in the hypoglossal motor nucleus, lateral tegmental field and ventral respiratory group were *extracellularly* recorded and *cross-correlated* with that of hypoglossal and phrenic nerves.

The theory underlying the electrophysiological techniques I used requires explanation and is detailed below. Experimental protocols are listed in each chapter.

### 2.1 Animal and Tissue Preparation

#### 2.1.1 Adult *In-vivo* Decerebrate Preparations

Adult, male Sprague-Dawley rats (Charles River) weighing between 300 and 350 grams were used and prepared as follows. They were anaesthetised with 2.0-2.5% Halothane in oxygen, and anaesthesia was maintained (2.0 - 1.0 %) until decerebration was complete. The trachea, femoral artery, and jugular veins were cannulated. The bladder was also cannulated because bladder distension in cats inhibits respiratory motor output (Gdovin et al.,
1994). In addition, urine production was used as an indication of the 'health' and stability of the animal. The phrenic, hypoglossal and vagal nerves were dissected. Depending on the experimental protocol, either left and right phrenic and hypoglossal nerves were dissected and cut, or the ipsilateral hypoglossal and phrenic were dissected and cut. Animals were then mechanically ventilated through the tracheal cannula with supplemental oxygen (~ 80 %) added to the inspiratory line. Expired carbon dioxide was monitored and maintained between 4 and 7 % (Datex, CD-107-27-02).

Atropine sulphate (0.05 mg) and dexamethasone sodium phosphate (0.2 mg) were given intramuscularly to minimise airway fluid secretion and brain oedema, respectively. A continuous infusion (2 ml 1M NaHCO₃ and 10 ml 5% Dextrose in 38 ml Ringer's) was established via the jugular vein (3-5 ml/kg/h) to stabilise the animal's fluid balance and help maintain femoral arterial blood pressure between 80-110 mmHg. Rectal temperature was monitored and maintained at 37 ± 1°C with a servo-controlled electric heating-pad (Harvard Apparatus, 30-7079).

The animals were held in a stereotaxic frame (Kopf) and by clamps (Kopf) at the rostral and sacral vertebrae with the head inclined forward at 30° (see figure 2.0). Decerebration was accomplished by the following procedure, and is illustrated in figure 2.1. Bilateral burr holes were made in the parietal skull plates, and vascular clips were then applied to the central sagittal sinus before removing the central portion of bone between the burr holes to establish a slot across the width of the skull approximately 5 mm wide. Brain tissue was then rapidly, but carefully aspirated (see figure 2.1). Bleeding was reduced by firmly applying small (~ 5 mm diameter) hand-made cotton balls soaked in thrombin to the ventral surface of the cranium. As much brain tissue as possible was removed in both the
Figure 2.0. A photograph of a stereotaxically restrained adult, decerebrate rat and the experimental apparatus used to record from respiratory nerves and neurones.
Figure 2.1. A photograph of the decerebration process. The cortex of an anaesthetised adult rat is gently removed by suction while small cotton balls soaked in thrombin are packed over floor of the skull.
rostral and caudal directions. To control bleeding, the surface of remaining brain tissue was covered with small pieces of Gelfoam® soaked in thrombin. In initial experiments, post-mortem analysis revealed that all tissue rostral to the precollicular level was removed. Halothane administration was then stopped, and animals were then paralysed with pancuronium bromide (intravenously at 0.4 mg/h).

In Chapters 3, 5, and 6, experimental protocols required exposure of the spinal cord and brainstem for making cellular recordings, stimulations and lesions. Exposure of the spinal cord and brainstem were accomplished by removing the muscles over and attached to the vertebrae and occipital skull, then by dorsal laminectomy on the first, second and third cervical vertebrae, and by removal of the occipital bone. The dura and pia mater were reflected. To gain easy access to the brainstem, the dorsal cerebellum was removed by suction.

Finally, the phrenic, hypoglossal and vagal nerves were further dissected, cut distally and placed in a paraffin oil pool for recording.

2.1.2 Adult Anaesthetised Preparations

Although I used the decerebrate preparation as a standard adult model, it was necessary to use anaesthetised rats on one occasion. According to the protocol in Chapter 5, it was necessary to keep adult rats in the supine position so that the medial and lateral branches of the hypoglossal nerve could be dissected, cut and recorded. However, since decerebration is accomplished with the rat fixed in a prone position in a stereotaxic apparatus, it was necessary to use anaesthetised rats.

Rats were anaesthetised with 2.0 - 2.5 % Halothane in oxygen. The trachea was
cannulated and animals were then mechanically ventilated with supplemental oxygen (~80 %) added to the inspiratory line. Atropine sulphate (0.05 mg) was given intramuscularly to minimise airway fluid secretion. Rectal temperature was monitored and maintained at 37 ± 1°C with a servo-controlled electric heating pad (Harvard Apparatus, 30-7079). They were bilaterally vagotomized, and the medial and lateral branches of one hypoglossal nerve were dissected, cut and prepared for whole-nerve recording.

2.1.3 Neonatal In-vitro Preparations

2.1.3.1 Brainstem-Spinal Cord Preparations

Under deep Halothane anaesthesia, 1-5 day-old Sprague-Dawley rats were decapitated at the fifth and sixth cervical spinal level, and the head was then immediately immersed in ice-cold artificial cerebral spinal fluid. Its composition in mM was 125 NaCl, 3 KCl, 1 KH₂PO₄, 2 CaCl₂, 1 MgSO₄, 25 NaHCO₃, and 30 D-glucose. The artificial cerebral spinal fluid was bubbled with 5 % CO₂ in O₂ to produce a pH of ~7.4.

After the skin and muscle over the skull and cervical spinal column were cut away, the parietal, and occipital bones were carefully removed, and a dorsal laminectomy performed from the first to fourth cervical vertebrae. The olfactory, cranial, and spinal nerves were cut and the whole brainstem-spinal cord then excised from the skull and vertebral column. To isolate the brainstem and cervical spinal cord both the cerebrum and cerebellum were removed. The resulting tissue constituted the brainstem-spinal cord preparation that is represented schematically in figure 2.2A.
Figure 2.2. A: a schematic representation of the in-vitro brainstem-spinal cord preparation. A 400-800 μm thick transverse slice at the level of two most rostral hypoglossal (XII) nerve rootlets constitutes the transverse brainstem-slice preparation (B). The pre-Bötzing complex, and XII motor nuclei are represented in the slice preparation.
2.13.2 Transverse Brainstem-Slice Preparations

These preparations were made by further reducing the brainstem-spinal cord preparation, and were made as follows. A brainstem-spinal cord was mounted on a block of agar by its dorsal surface (cyano-methacrylate) and placed rostral end down. Thin slices were then cut using a vibratome (752M Vibroslice, Campden Instruments Inc.) until the two most rostral hypoglossal nerve rootlets were seen. At this point, a thin 400-800 μm slice incorporating the nerve rootlets was made and constituted the transverse brainstem slice represented schematically in figure 2.2B. The remaining dura mater covering the underlying tissue was removed.

2.13.3 In-Vitro Tissue Maintenance

To maintain the tissue viable for recording conditions, both brainstem-spinal cord and transverse brainstem-slice preparations were transferred into a custom-built recording chamber perfused with artificial cerebral spinal fluid at 15-20 ml/min, where the tissue stabilised for 30 min at 20-25°C (figure 2.3). During this time, potassium chloride concentration was increased from 3 to 8 mM to establish and maintain a stable respiratory motor output (Ramirez et al., 1996; Smith et al., 1991). Under these conditions, a stable respiratory-like rhythm could be recorded for at least 4-5 hours.
2.2 Overview of Experimental Instrumentation

Figure 2.0 and 2.3 are photographs of the experimental set-up used for in-vivo and in-vitro experiments, respectively. While the physical recording set-up used for in-vivo and in-vitro experiments appears different, the experimental instrumentation used to record from nerves and neurones are identical, and are outlined in figures 2.4, 2.5, 2.6 and 2.7.

2.3 Nerve and Cellular Recordings and Stimulation

2.3.1 Hypoglossal and Phrenic Nerves

As outlined above, the instrumentation used to record from hypoglossal and phrenic nerves was identical for both in-vitro and in-vivo rats. The only difference being that in in-vitro preparations, nerve rootlets were recorded using suction electrodes whereas in-vivo preparations, these nerves were recorded with bipolar silver electrodes. Phrenic and hypoglossal nerve electrode signals were amplified (Neurolog, NL104), filtered (bandpass 0.12-8 kHz) with emphasis around 1 kHz using a 10-band stereo equaliser (Audio Reflex, EQ-1) and integrated (time constant = 50 ms; Neurolog, NL703) (see figures 2.4 and 2.5).

2.3.2 Extracellular Recordings

Extracellular recordings were made using glass-microelectrodes (0.3-0.5 MΩ at 1 kHz). Microelectrode signals (Axon Instruments, Axoprobe-1A) were amplified (Neurolog, NL106) and filtered (bandpass 0.1-20 kHz; Neurolog, NL126) (figure 2.6). During initial identification, extracellular recordings were made at wide bandwidths (0.01-20 kHz) to help distinguish axons from neurones (e.g., monopolar vs. bipolar action potentials) and then were either filtered at limited bandwidths to obtain the best spike discrimination or were left
Figure 2.3. A photograph of the experimental set-up used to record the respiratory-like activity of in-vitro preparations. Preparations are placed in a tissue chamber and whole-nerve suction electrodes are used to record respiratory-related activity; electrodes are positioned using a micro-manipulator and nerve rootlets are visualised with a light/fluorescence microscope. aCSF, artificial cerebral spinal fluid.
Figure 2.4. A schematic diagram of the electrophysiological instrumentation used to record and process the respiratory activity monitored on the phrenic (C4) nerve.
Figure 2.5. A schematic diagram of the electrophysiological instrumentation used to record and process the inspiratory activity of the hypoglossal (XII) nerve.
Figure 2.6. A schematic diagram of the electrophysiological instrumentation used to record and process the extracellular activity of inspiratory neurones.
Figure 2.7. A schematic diagram of the electrophysiological instrumentation used to record the intracellular activity of inspiratory hypoglossal (XII) motoneurones.
unfiltered. Microelectrodes were stereotaxically positioned relative to the obex using a micromanipulator (Canberra, Narishige), and advanced into the tissue using a microstepper (Digitimer, Significat) (figure 2.6).

2.3.3 Intracellular Recordings

Intracellular glass-microelectrodes (filled with 3 M potassium chloride or 2 M potassium citrate; 5-15 MΩ at 1 kHz) used to record hypoglossal motoneurones were made from microfilament glass tubing (A-M Systems) using a vertical pipette-puller (Kopf Model 750). Intracellular signals (Axon Instruments, AxonProbe 1A) were amplified (Neurolog, NL106) and filtered (0.01-20 kHz; Neurolog, NL126) (figure 2.7).

2.3.4 Stimulation

Both hypoglossal and vagus nerves were stimulated via silver bipolar recording electrodes with pulses (0.2 ms, 2-10 V, 5 Hz) derived from an isolated stimulator (Digitimer, DS2) driven by a stimulus programmer (Digitimer, 4030).

Glass-coated tungsten microelectrodes (0.5-1.1 MΩ) were used for antidromic and orthodromic activation of inspiratory neurones and spinal funiculi. Stimulating microelectrodes were positioned using a micromanipulator (Narishige). Stimulus pulses (0.2 ms, 10-25 µA, 5 Hz) were obtained from a stimulus isolator (Digitimer, Model DS2) driven by a stimulus programmer (Digitimer, D4030) via a pulse buffer (Neurolog, NL510).
2.3.5 Signal Display and Storage

All signals were displayed on oscilloscopes (Tektronics, Nicolet), monitored with loudspeakers and digitally stored (Vetter) on videotape and on a PC computer. A chart recorder (Graphtec, WR3600) provided permanent records of signals.

2.4 DESCRIPTION OF EXPERIMENTAL PROCEDURES

2.4.1 Cross-Correlation Analysis

Cross-correlation histograms are used to demonstrate synchronous events between individual neurones or whole nerves thereby inferring synaptic connexions, and the technique is illustrated in figure 2.8. Synaptic connexions can be deduced based on the following statistical theory: if two spike trains are independent, that is, if the occurrence of a spike in one neurone (or nerve) is not correlated with the activity of another neurone (or nerve), and vice versa, the cross-correlation histogram is flat, that is, the cross-correlation function is constant (Kirkwood, 1979; Kirkwood and Sears, 1991; Moore et al., 1970). When the cross-correlation histogram is not flat this is indicative of some temporal correlation between these neurones (or nerves). It should be understood that short-term synchronisation of events might be due to either cause or effect or a common input from presynaptic sources. Nevertheless, meaningful interpretations about neuronal connexions can be inferred from cross-correlation histogram features. The presence of peaks (excitation) or troughs (inhibition) in the histogram indicates probabilities of firing greater or less than expected by chance and thus reveals functional connexions between neurones (Kirkwood, 1979; Moore et al., 1970; Vachon and Duffin, 1978). The attributes of cross-correlation histogram features can be assessed to determine whether synaptic input originates from common, monosynaptic
Figure 2.8. A schematic representation of cross-correlation analysis. This example illustrates cross-correlation of the extracellularly activity of a hypoglossal premotor neurone (XII Pre-Mn) and the XII nerve. An action potential generated in the neurone ‘triggers’ the cross-correlation process; at a latency, which is due to synaptic delay and transmission time, the hypoglossal (XII) nerve discharges, and a ‘peak’ begins to grow in the cross-correlogram.
or polysynaptic inputs. Common excitation of neurones results in cross-correlation histograms (cross-correlograms) that exhibit a broad central peak; common inhibition also results in a broad central peak (Moore et al., 1970). Monosynaptic excitation results in peaks with narrow half-amplitude widths (~1.5 ms) at a short latency (>0.2 ms and <2 ms). Polysynaptic excitation results in similar features but at longer latency and wider half-amplitude widths. These criteria were used to examine cross-correlogram features and are derived from interpretations made by Kirkwood (Kirkwood and Sears, 1991), and were also tested by the recently developed cross-correlation stimulation program developed in this laboratory (Duffin, 2000).

For computing cross-correlograms, inspiratory neurone action potentials were discriminated with time-amplitude window gates (Bak), and phrenic and hypoglossal nerve action potentials were amplitude gated (Bak). Cross-correlograms were computed on-line using both a hard-wired cross-correlator (Anderson and Duffin, 1976) as well as a computer; discriminated pulses were input via an A/D interface (AT-MIO-16XE-10, National Instruments), where specially written software (National Instruments, LabVIEW), simultaneously cross-correlated neurone activity to the inspiratory discharge of both hypoglossal and phrenic nerves.

2.4.2 Antidromic Activation

This electrophysiological technique was used to discover axonal projections of a neurone, and is illustrated schematically in figure 2.9. A neurone of interest was extracellularly or intracellularly recorded and a stimulating electrode was used to antidromically activate the recorded neurone by stimulation, and thereby demonstrate that the
A hypoglossal motoneurone (XII Mn) is extracellularly recorded and is antidromically activated by stimulation (pulse width, 0.2 ms, intensity ~2 V) of the XII nerve. ‘Collision’ of an evoked action potential and a spontaneous generated action potential verifies that the recorded neurone sends an axon to the location of the stimulating electrode.
neurone projects to the location of the stimulating electrode (Lipski, 1981). For example, stimulation of the hypoglossal nerve while extracellularly recording an inspiratory neurone within the stereotaxically defined hypoglossal motor nucleus (Paxinos and Watson, 1986) generates antidromic action potentials in the descending axon of the cell. To ensure that it was in fact the axon of the recorded neurone that was activated and not, for example, an ascending afferent, which synaptically excited the neurone, a collision test was performed (Lipski, 1981). A collision test is designed to demonstrate that an evoked action potential and a spontaneous action potential can be 'collided' (see figure 2.9), which indicates that the stimulated axon comes from the recorded neurone soma.

2.4.3 Chloride Iontophoresis

This technique is used to determine whether an intracellularly recorded neurone is postsynaptically inhibited (Tian et al., 1998c; Withington-Wray et al., 1988; Woch and Kubin, 1995). Postsynaptic inhibition is a chloride-dependent process (Woch and Kubin, 1995). When a neurone is actively inhibited, chloride ions flow down their concentration gradient and enter the neurone causing it to hyperpolarize. However, injection of chloride ions reverses this process, so that chloride-mediated hyperpolarization is manifested as a depolarisation.

2.5 Data and Statistical Analysis

2.5.1 Cross-Correlograms

Features observed in the cross-correlograms were quantified using the $k$-ratio (Sears and Stagg, 1976). For peaks, the $k$-ratio was calculated by dividing the peak bin count by the mean bin count; for troughs, the sum of the mean bin count and the difference between the
mean bin count and the nadir for the trough divided by the mean bin count determined the \( k \)-ratio. The mean bin count was measured away from any features present in the histogram. Peaks and troughs were tested for statistical significance at \( P < 0.01 \) or 0.001 levels (Graham and Duffin, 1981); only features shown to be statistically significant are reported.

### 2.5.2 Respiratory Variables

In both in-vivo and in-vitro preparations, ten consecutive respiratory cycles were analysed and averaged to determine each variable, and all measurements were made from the raw neurograms. I measured the total cycle length (defined as the period between the beginning of one inspiration and the beginning of the next), inspiratory period (the start to finish of an inspiratory burst), and expiratory period (the end of an inspiration to the start of the next). Respiratory frequency was determined as the inverse of averaged total cycle length.

In Chapter 5 and 6, I measured the different inspiratory onset times of hypoglossal and phrenic nerve discharges from raw neurograms. In Chapter 6, the rate of rise of inspiratory activity was assessed; the time taken for the integrated inspiratory discharge to reach 80% of the peak value was expressed as a percentage of the inspiratory period.

Except for statistical analysis of cross-correlograms (described above, section 2.5.1), the statistical tests used to analyse respiratory timing variables is outlined in the Methods section of Chapter 3-6.
CHAPTER 3

THE BILATERAL RESPIRATORY RHYTHM-GENERATING NETWORK

3.0 SUMMARY

In mammals, respiratory neurones in the brainstem are arranged in longitudinally distributed groups that are duplicated on each side of the neuraxis. My aim was to determine whether each side generates and transmits respiratory rhythm independently. To do this, I recorded respiratory motor output while making mid-sagittal sections of the medulla in both adult and neonatal rat preparations. In the decerebrate adult rat, the respiratory activities of left and right phrenic nerves became asynchronous, after mid-sagittal section of the medulla; however, transection of the medulla, in the in-vitro brainstem-spinal cord and transverse brainstem-slice preparations abolished the expression of respiratory rhythm. From these observations, I suggest that in adult rats, each half of the medulla contains sufficient neural circuitry to independently generate and transmit respiratory rhythm, but in neonatal rat preparations, it does not.

3.1 INTRODUCTION

The neural network generating respiratory rhythm is located in the medulla; the neurones that compose this network are configured in bilateral columns running parallel to the neuraxis, in close proximity to the nucleus ambiguus. From this symmetrical arrangement, I hypothesise that respiratory rhythm may be generated independently by each bilateral column. Previous experiments to separate the two sides of the medulla and test this
hypothesis by recording the activity of left and right phrenic nerves have produced conflicting results.

In the adult rabbit and monkey, left and right phrenic nerve activities become asynchronous after a mid-sagittal section of the medulla (Gromysz and Karczewski, 1981; Gromysz and Karczewski, 1982; Janczewski and Karczewski, 1984; Karczewski and Gromysz, 1982). However, in adult cats, this same procedure abolishes bilateral phrenic nerve activity (Gromysz and Karczewski, 1982; Gromysz and Karczewski, 1984; Kubin et al., 1987; St.-John, 1983). It might be concluded that breathing in the rabbit and monkey is controlled by two parallel, independent networks of respiratory neurones, which are able to generate respiratory rhythm in the absence of cross-connexions whereas cross-connexions in adult cats are essential for respiratory rhythm generation. However, I suggest that this apparent difference between species may not be due to differences in medullary organisation, but due to differences in the pathways connecting medullary inspiratory premotor neurones to respiratory motoneurones. In the cat, medullary inspiratory neurones project mainly unilaterally to the contralateral phrenic motoneurones, and since these pathways cross at the medullary level, sagittal sectioning would interrupt these connexions (Ezure, 1990; Long and Duffin, 1986; Merrill, 1970). In the adult rat, medullary inspiratory premotor neurones project bilaterally to both ipsilateral and contralateral phrenic motoneurones (see figure 3.0) (Duffin and van Alphen, 1995a; Lipski et al., 1994; Tian and Duffin, 1996b). I therefore hypothesised that a mid-sagittal transection of the rat medulla would spare the ipsilateral connexions between medullary inspiratory neurones and phrenic motoneurones. Accordingly, I observed the effects of mid-sagittal lesion of the medulla in in-vivo, adult and
Figure 3.0. A schematic representation of the known transmission pathway from medullary inspiratory premotor neurones to phrenic motoneurones in the spinal cord in the adult rat. Ventral respiratory group inspiratory premotor neurones bifurcate to descend both left and right sides of the spinal cord in adult rats.
in-vitro neonatal rat preparations on respiratory activity, to determine if respiratory rhythm is generated independently on each side of the medulla.

The results obtained from adult rat preparations have been published (Peever et al., 1998).

3.2 METHODS

3.2.1 Animal Preparation

In-vivo Preparations: Adult male Sprague-Dawley rats weighing 350-500 grams were prepared by the methods described in Chapter 2, section 2.1.1.

In-vitro Preparations: Neonatal rats (2-3 day-old) were used to prepare both in-vitro brainstem-spinal cord and transverse brainstem-slice preparations (see Chapter 2, section 2.1.3.).

3.2.2 Protocol

3.2.2.1 In-vivo Preparations

The respiratory activity of left and right phrenic nerves was recorded while lesions along the midline of the medulla were made. Using the obex as a reference co-ordinate, mid-sagittal sections were made using a micro-scalpel blade (Fine Science Tools Inc.). The blade was held in a micromanipulator (Narishige, Canberra) and positioned 0.5 mm lateral of the mid-line in order to avoid the vertebral (dorsal surface) and basilar (ventral surface) arteries on the mid-line of the brainstem and spinal cord. The blade was advanced slowly from 3.5 mm rostral to 3.5 mm caudal of obex in 500 μm ventral increments until the scalpel blade touched the ventral surface of the vertebral column. At the end of each experiment, the rat brainstem and cervical spinal cord were removed and fixed in 4 % paraformaldehyde for at
least 24 hours. After fixation, the lesion site was visually examined to verify that the brainstem was completely sectioned.

3.2.2.2 In-Vitro Preparations

Mid-sagittal sections were made using a micro-scalpel blade (Fine Science Tools Inc.), which was held in a micromanipulator (Narishige, Canberra) and positioned along the mid-line of the tissue preparation at specific anatomical landmarks (see figure 3.1).

In brainstem-spinal cord preparations, either phrenic or hypoglossal nerve rootlet activity was recorded. Two series of mid-sagittal lesions were performed: (1) those that extend from the most caudal phrenic nerve rootlet to the most caudal hypoglossal nerve rootlet (figure 3.1Aa); and (2) those that extended from the most caudal to rostral hypoglossal nerve rootlets (figure 3.1Ab). When phrenic nerve activity was recorded both series of midline lesions were performed, but when hypoglossal nerve rootlet activity was recorded only the lesion extending from the caudal to rostral hypoglossal nerve rootlets was performed.

In transverse brainstem-slice preparations, a mid-sagittal transection of the medulla was made from the ventral surface of the slice to the ventral region of the hypoglossal motor nuclei (see figure 3.1B).

3.2.3 Data Analysis

In in-vivo adult rats, phrenic nerve discharge amplitude data were acquired from phrenic neurograms and are expressed as per cent change with the range of values included.
Figure 3.1. A and B are schematic representations of the in-vitro brainstem-spinal cord and transverse brainstem-slice preparations, and the dotted lines illustrate the mid-sagittal transections performed. XII, hypoglossal.
Means ± standard error were not used as the phrenic nerve discharge amplitudes cannot be compared between animals due to differences in recording conditions (that is, electrode contact and different amplifications).

In *in-vitro* preparations, mid-sagittal transections resulted in tissue movement, which caused nerve rootlets to be pulled out of the recording electrodes. Therefore, only the frequency of inspiratory bursting was measured because recording conditions were not constant before and after the transections.

### 3.2.4 Statistical Analysis

All data, excluding inspiratory amplitude (adults) are expressed as mean ± standard error. Significant differences between means were assessed by paired *t*-tests with the level of significance considered at $P < 0.05$.

### 3.3 RESULTS

#### 3.3.1 In-Vivo Preparations

Although sections were made 0.5mm lateral of mid-line to avoid cutting both basilar and vertebral arteries, in 4 rats the basilar artery was cut and excessive bleeding resulted, leading to a loss of respiratory activity, and these data are not included here. Successful, mid-sagittal sections were performed in a total of 11 rats; in 5 rats, the phrenic nerve was recorded on one side only, and in 6 rats both sides were recorded, and it is these data, which are presented herein. A visual inspection of the brainstems after fixation showed that they had been completely separated in all cases.
Sectioning never abolished phrenic nerve activity (n = 11). In the 6 rats where both left and right phrenic nerve discharge was recorded, the nerve discharges were synchronous before sectioning but asynchronous after sectioning (figure 3.2). In all cases, asynchronous activities were observed only when the section had reached a point approximately 1 mm caudal to the obex.

Sectioning of the brainstem affected both phrenic nerve discharge amplitude and burst duration. In the 6 rats where both left and right phrenic nerves were recorded I observed the following changes. When the section reached a depth below the dorsal surface of 1.5 mm, burst amplitude had decreased significantly (P = 0.027) in the left phrenic by 13% (range: 3% increase to a 20% decrease) and decreased (not significantly, P = 0.183) in the right phrenic nerve by 7% (range: 15% increase to a 18% decrease) with no significant difference between them (P = 0.06). When the section reached a depth of 1.5 mm below the dorsal surface burst duration had significantly increased (P = 0.009) to 0.66 ± 0.17 s from 0.38 ± 0.13 s before sectioning, and in the right phrenic nerve burst duration significantly increased (P = 0.036) to 0.66 ± 0.18 s from 0.42 ± 0.13 s before sectioning with no significant difference between the left and right phrenic nerves either before (P = 0.50) or after (P = 1.0) the section.

After complete sectioning of the brainstem different rhythms were observed in left and right phrenic nerve discharges. Compared to the intact brainstem, burst amplitude significantly decreased (P = 0.002) in the left phrenic nerve by 81% (range: 65% to 92% decrease) and in the right phrenic nerve by 65% (P = 0.01) (range: 21% to 88% decrease) with no significant difference between them (P = 0.892). Burst duration returned to values not significantly different from those observed before sectioning, 0.39 ± 0.18 s (P = 0.210)
Figure 3.2. Mid-sagittal section of the brainstem in a decerebrate rat. Right and left phrenic nerve discharge at a sectioned depth of 2.5 mm (upper) and 5 min after complete section (lower). The inset shows left phrenic discharge before sectioning.
in the left phrenic nerve and 0.49 ± 0.12 s (P = 0.625) in the right phrenic nerve with no significant difference (P = 0.593) between left and right.

Sectioning of the medulla had no clear effect on phrenic burst frequency. Before sectioning, burst frequency was 30 ± 9 b/min; the lesion made 1.5 mm below the dorsal surface of the brainstem had no significant effect on bursting frequency (P = 0.130). After complete section of the brainstem burst frequency in left phrenic nerve was 29 ± 15 b/min, and in the right phrenic nerve was 36 ± 15 b/min with no significant change in either side compared with control values. There was no significant difference between the left and right sides after complete section of the brainstem (P = 0.290).

3.3.2. In-Vitro Preparations

Brainstem-spinal cord preparations: The effects of mid-sagittal sections of the spinal cord and medulla on phrenic nerve bursting frequency were examined in 6 preparations, and an example of one recording is illustrated in figure 3.3A. Prior to lesions, bursting frequency was 6.1 ± 0.50 b/min. Lesions that extended from the caudal phrenic nerve rootlets to the caudal hypoglossal nerve rootlets had no effect on phrenic bursting frequency (6.2 ± 0.51 b/min; P = 0.986) (figure 3.3Ab), however, transection between the caudal to rostral hypoglossal nerve rootlets caused respiratory rhythm to stop (see figure 3.3Ac). In 2 of the 6 preparations, phrenic nerve rootlets remained within the suction electrodes during the lesions and respiratory motor output was recorded during the transection. In these preparations, I observed that phrenic activity stopped when the scalpel blade reached the level of the two most rostral hypoglossal rootlets.
Figure 3.3: A: the respiratory-related activity of phrenic nerve discharge recorded from a brainstem-spinal cord preparation (a); phrenic nerve discharge after a midline transection that extends from the phrenic motor nuclei level of the spinal cord to the most caudal hypoglossal rootlet in the medulla (b); phrenic nerve discharge is abolished following a midline transection that extends from the most caudal to rostral hypoglossal rootlets (c). B: respiratory-related activity of the hypoglossal nerve before (a) and after (b) midline transection of the medulla from the most caudal to rostral hypoglossal rootlets.
The effects of mid-sagittal transection of the medulla at the level of the hypoglossal nerve rootlets on hypoglossal nerve bursting frequency were examined in 6 preparations, and an example of one recording is shown in figure 3.3B. Before the preparations were lesioned, hypoglossal nerve bursting frequency was $6.5 \pm 1.5$ b/min, however complete mid-sagittal transection of the medulla at the level of the caudal to rostral hypoglossal nerve rootlets caused respiratory rhythm to stop (figure 3.3B).

*Transverse Brainstem-Slice Preparations:* The effects of mid-sagittal transection of the medulla on hypoglossal nerve bursting frequency were examined in 4 preparations, and an example of one recording is shown in figure 3.4A/B. Before the transection, bursting frequency was $6.5 \pm 0.65$ b/min, however, following mid-sagittal transection respiratory rhythm was abolished (figure 3.4B).

### 3.4 Discussion

From the observations reported above, I draw two conclusions about the bilateral respiratory network in the rat: (1) in the adult, each half of the medulla is capable of generating and transmitting respiratory rhythm independently; and (2) in *in-vitro* neonatal preparations, each side of the medulla does not contain sufficient neural circuitry to independently generate and/or transmit respiratory rhythm. These findings will be discussed in light of our current understanding of respiratory rhythm generation and its transmission in both adult and neonatal rats.

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Figure 3.4. The respiratory-related activity of hypoglossal nerve discharge in a transverse brainstem-slice preparation before (A) and after (B) a midline transection of the slice preparation.
3.4.1 Respiratory Rhythm Generation

In adult rats, phrenic nerve activity was maintained in both left and right nerves but became asynchronous after complete section of the medulla, 3.5 mm above and 3.5 mm below the obex level. These results are therefore in agreement with previous observations in both the rabbit and monkey (Budzinska and Romaniuk, 1985; Gromysz and Karczewski, 1981; Gromysz and Karczewski, 1982; Gromysz and Karczewski, 1984; Karczewski and Gromysz, 1982; Kubin et al., 1987), but differ from most of those made in the cat.

Although the majority of investigations in cats have reported that complete mid-sagittal section of the medulla leads to the cessation of phrenic nerve activity (Gauthier et al., 1986; Gromysz and Karczewski, 1982; Gromysz and Karczewski, 1984; St.-John, 1983), some have reported that phrenic nerve activity is preserved in hypercapnia; but the left and right nerve discharge remains synchronised after sectioning (Gromysz and Karczewski, 1982; Gromysz and Karczewski, 1984). In contrast, one group has shown that sectioning leads to asynchronous left and right phrenic nerve activities (Eldridge and Paydarfar, 1989). In support of this latter finding, others have demonstrated that complete medullary section results in asynchronous left and right hypoglossal and recurrent laryngeal nerve activities (Kubin et al., 1987; St.-John, 1983).

Progressively deeper sectioning, from 3.5 mm rostral to 3.5 mm caudal of the obex, resulted in a progressive decrease in phrenic amplitude. I interpreted this finding as indicating that medullary cross-connexions may be involved in maintaining respiratory drive (Budzinska and Romaniuk, 1985; Eldridge and Paydarfar, 1989). Because phrenic nerve activity did not disappear, I suggest that these cross-connexions are not essential in the
maintenance of respiratory activity, but rather function to distribute the respiratory drive originating from each half of the brainstem (Eldridge and Paydarfar, 1989).

Although phrenic amplitude decreased as the section was made more ventral, asynchrony of left and right phrenic nerve activities did not occur until the section was made approximately 1 mm caudal to the obex, similar to observations in the rabbit (Gromysz and Karczewski, 1981). In cats, lesions of 2.5-4 mm caudal to the obex are required to produce asynchronous activities in left and right recurrent laryngeal (Kubin et al., 1987; St.-John, 1983) and phrenic nerves (Eldridge and Paydarfar, 1989). The difference in lesion dimensions between cat and rat/rabbit is probably due to the significantly larger brainstem in cats as compared with rat and rabbit. Independent respiratory rhythm generation in left and right halves of the brainstem therefore appears to result from the loss of cross-connexions at the caudal end of the obex. I suggest that at this level cross-connexions between respiratory neurones are responsible for synchronising the left and right sides of the respiratory rhythm-generating network.

These data clearly demonstrate that each half of the bilateral respiratory network in the adult rat is capable of independently generating respiratory rhythm, and that cross-connexions between the left and right sides are required to synchronise the respiratory activity of bilaterally situated phrenic motoneurones. Although the underlying mechanisms of bilateral synchronisation are unknown and require further understanding, I nonetheless suggest that future analysis of the fundamental mechanisms underlying rhythm-generation may be confined to only one side of the medulla in adult rats.

In the in-vitro brainstem-spinal cord preparation, mid-sagittal transection of the spinal cord and caudal medulla had no effect on phrenic nerve rootlet discharge. This observation
is in agreement with that reported by others (Harada et al., 1985; Onimaru et al., 1989). Unlike adult rats, mid-sagittal transection of the medulla abolished the expression of respiratory-related rhythm on both phrenic and hypoglossal nerve rootlets in both the in-vitro brainstem-spinal cord and transverse brainstem-slice preparations. However, in 2 cases, I observed that rhythm was abolished when the mid-sagittal transection reached the level of the most rostral hypoglossal nerve rootlets, a region that roughly corresponds to the obex, and from where transverse brainstem-slice preparations are made. I conclude that at this level essential cross-connexions or midline neural structures (e.g. nucleus raphé obscurus) are severed or destroyed, which either disrupts rhythm-generation or eliminates its expression.

These results are consistent with those reported in in-vivo and in-vitro preparations from neonatal rats. In a recent abstract, Janczewski and Aoki (1997) report that mid-sagittal transection of the medulla abolished inspiratory activity of diaphragmatic contractions in 2-9 day-old intact, anaesthetised rats. Similarly, in in-vitro brainstem-spinal cord preparations from 0-4 day-old rats, McLean and Remmers (1994) report that mid-sagittal transection of the medulla stopped the expression of the respiratory-related discharge of phrenic nerve rootlets. These observations are also consistent with unilateral lesions studies, which reveal that focal destruction or disruption of cellular activity within the rostral ventral respiratory group abolishes the expression of respiratory rhythm in both in-vitro brainstem-spinal cord and transverse brainstem-slice preparations from neonatal rats (Funk et al., 1993; Onimaru et al., 1988).

The discrepancy between adult and neonatal preparations has several possible explanations. Although neuroanatomical tracing evidence (Ellenberger, 1999) demonstrates
that the overall configuration of the neonatal respiratory network is organised similarly to that in the adult rat, Hilaire et al. (1990) report the absence of rhythmically active respiratory neurones in the dorsal respiratory group of in-vitro brainstem-spinal cord preparations from 0-3 day-old rats. As previously suggested (Duffin et al., 1995; Ramirez et al., 1996), the respiratory network may be subject to developmental change, and although the general topology of respiratory neurones is similar in adult and neonatal rats, the underlying interconnexions and/or mechanisms of rhythm generation may differ. Therefore, unlike adult rats, both halves of the medulla may be required to generate the rhythm in neonatal rats. However, in a recent abstract, Johnson et al. (2000) report that pacemaker neurones within the putative rhythm-generating pre-Bötzinger complex remain rhythmically active when isolated from the transverse brainstem-slice preparation. Based on their preliminary observations, it might be suggested that each half the neonatal respiratory network is capable of generating rhythm independent of the other half.

Another explanation for the loss of rhythm following mid-sagittal transection is that sufficient respiratory drive is eliminated during the transection such that respiratory rhythm is no longer expressed, despite its continued generation. Not only is this contention consistent with the observation of Johnson et al. (2000), it is also consonant with observations from this laboratory. Recently, I found that disrupting the cellular activity of nucleus raphé obscurus by micro-injection of kainic acid (4.5 mM), transiently eliminated the expression of respiratory-related hypoglossal nerve rootlet activity in transverse brainstem-slice preparations from neonatal rats (Peever et al., in press). I also found that focal CO₂-acidification of this midline region significantly increased the amplitude, frequency and duration of hypoglossal nerve rootlet inspiratory bursting. From these observations, I suggest
that the midline nucleus raphé obscurus is an integral component of the *in-vitro* neonatal respiratory network, which provides an excitatory input to both the pre-Bötzinger complex and hypoglossal motor nuclei. It is therefore possible that mid-sagittal medullary transection sufficiently disrupts the excitatory inputs from the midline raphé nuclei such that rhythm is no longer expressed in the *in-vitro* preparations. This hypothesis is supported by observations from adult cats, which showed that mid-sagittal transection initially abolished the expression of rhythm, but increased drive resulting from severe hypercapnia reinstated it (Gromysz and Karczewski, 1984). Alternatively, I suggest that although each side of the medulla continually generates respiratory rhythm, it is not expressed because the *transmission pathways* from the generator to respiratory motoneurones have been severed.

### 3.4.2 Respiratory Rhythm Transmission

While the experiments presented herein offer no specific details of individual neuronal connexions, it is postulated that respiratory rhythm-generating neurones *per se* must be bilaterally duplicated because respiratory rhythm continues to be generated and expressed following mid-sagittal medullary transection. Although the *functional* interconnexions between bilateral rhythm-generating neurones are unknown, the functional connexions between respiratory ventral respiratory group premotor and phrenic motoneurones are well documented (Monteau and Hilaire, 1991; Tian et al., 1998c). In adult rats, inspiratory and expiratory medullary premotor neurones are bilaterally duplicated and have axons that bifurcate to synchronically excite phrenic motoneurones located in both left and right phrenic motor pools (see figure 3.0) (Tian et al., 1998a; Tian and Duffin, 1996c; Tian et al., 1998c). Whether other respiratory premotor pathways, namely hypoglossal motoneurones, are
bilaterally duplicated is unknown in rats, and this will be investigated in Chapter 4. In adult cats, it is hypothesised that both phrenic and hypoglossal motoneurones receive respiratory drive transmission from a common premotor pathway (Ono et al., 1994; Ono et al., 1998). Indeed, if the transmission pathway to phrenic and hypoglossal motoneurones is via a common premotor population then respiratory rhythm should be preserved in both nerves following transection. However, in adult cats, mid-sagittal medullary transection eliminated the expression of respiratory rhythm on phrenic nerves but not on hypoglossal nerves (St.-John, 1983). Therefore, it may be hypothesised that phrenic and hypoglossal motoneurones do not share a common premotor pathway, as previously suggested by Morin et al. (1992) and Sica et al. (1984). This issue will be examined Chapter 5.

Unlike adult rats and cats, the premotor neurones that transmit respiratory drive to respiratory motoneurones are unknown in neonatal rats. Because mammals breathe at birth, it is logical to assume that the fundamental interconnexions within the central respiratory network are intact. However, recent evidence demonstrates that premotor pathways undergo marked postnatal development (Glover, 2000). Therefore, it is possible that the premotor pathways that transmit respiratory rhythm to respiratory motoneurones also undergo postnatal changes. The preliminary observations of Janczewski and Aoki (1997, 1998) indirectly support this claim. They found that in 2-9 day-old rats, diaphragmatic activity was eliminated following mid-sagittal transection of the medulla, but found that respiratory rhythm continued to be expressed in 17-22 day-old rats after the same lesion was made. Although splitting the medulla may not stop respiratory rhythm-generation, it might eliminate its expression because the premotor pathways that transmit rhythm have been severed, and I contend that unlike adult rats, only contralateral premotor pathways are
established in the neonatal rat. This hypothesis will be tested in the following chapter.

3.4.3 Comparison of \textit{In-vivo} and \textit{In-vitro} Preparations

In these experiments, I compared the nerve activities from \textit{in-vivo} adult rats with those of two \textit{in-vitro} preparations from neonatal rats. The \textit{in-vitro} preparations generate periodic bursts of neural activity on phrenic and hypoglossal nerve rootlets (Koshiya and Smith, 1999b; Ramirez et al., 1996; Smith et al., 1991; Smith et al., 1990; Suzue, 1984), and contain respiratory neurones with firing patterns similar to those of the ventral respiratory group in adult rats; including pre-inspiratory, inspiratory, expiratory and phase-spanning neurones (Onimaru and Homma, 1992; Onimaru et al., 1992; Ramirez et al., 1996; Smith et al., 1990). Further, these preparations detect and respond to pH changes in a manner similar to those seen in intact rats (Issa and Remmers, 1992; Johnson et al., 1997; Peever et al., 1999b), and when their temperature is increased to a more physiological level, the frequency and pattern of inspiratory discharge of both phrenic and hypoglossal nerves approaches that of intact age-matched neonatal rats (Peever et al., 1999b).

While some investigators accept the use of these \textit{in-vitro} preparations as models of intact neonatal rats with respect to rhythm generation and transmission (Smith et al., 1991; Smith et al., 1990; Telgkamp and Ramirez, 1999) others do not (Fung et al., 1994; St.-John, 1998; St.-John and Paton, 2000). Certainly the reduced preparations are relatively hypoxic (Okada et al., 1993) and at lower temperatures (26-29°C) than intact neonates, although the transverse brainstem-slice preparation (Koshiya and Smith, 1999b; Ramirez et al., 1996; Smith et al., 1991) offers superior control of the extracellular environment compared to the brainstem-spinal cord preparation (Ballanyi et al., 1992; Hayashi and Lipski, 1992).
3.4.4 Conclusions

In adult rats, separation of the bilateral respiratory rhythm-generating network by mid-sagittal transection produces asynchronous respiratory rhythms in both phrenic nerves. I conclude that each half of the respiratory network contains sufficient neural circuitry to independently generate and transmit respiratory rhythm to phrenic motoneurones.

In *in-vitro* neonatal preparations, mid-sagittal transection of the medulla eliminates the respiratory-related activity of phrenic and hypoglossal nerve rootlets. While it might be concluded that each side of the medulla cannot independently generate rhythm in these preparations, I contend that the premotor pathways from the respiratory rhythm-generating network to phrenic and hypoglossal motoneurones have been disrupted so that rhythm is no longer expressed. I speculate that there are postnatal changes in the functional connectivity between respiratory premotor neurones and motoneurones.
CHAPTER 4

BILATERAL SYNCHRONISATION OF RESPIRATORY MOTOR OUTPUT

4.0 SUMMARY

The short-term synchronisation of the discharges recorded from left and right phrenic nerves in the adult rat is produced partly by a shared excitation from a common premotor neurone population, and partly by short-term synchronisation of premotor neurones. However, the former synchronisation has not been examined for hypoglossal motoneurones in adult rats, or for phrenic and hypoglossal motoneurones in neonatal in-vitro preparations. In adult rats, cross-correlograms computed between the inspiratory discharges of the left and right phrenic nerves, and the left and right hypoglossal nerves displayed central peaks with half-amplitude widths of $1.4 \pm 0.1$ ms and $1.7 \pm 0.1$ ms (mean ± standard error), respectively. I interpreted them as evidence for common excitation. However, such central peaks were absent in the same cross-correlograms computed for neonatal in-vitro preparations; although central peaks were observed in cross-correlograms computed between the discharges recorded from adjacent phrenic nerve rootlets. I conclude that in the adult rat left and right hypoglossal nerve discharges are synchronised (over 1-2 ms) in part by excitation from a common premotor neurone population, as for the phrenic nerves, but this type of short-term synchronisation is undetectable in neonatal in-vitro preparations. I speculate that the differences between the adult and neonatal preparations are due to development of respiratory drive transmission pathways.
4.1 INTRODUCTION

For the most part, the mammalian central nervous system is bilaterally duplicated. Symmetry about the vertebrate neuraxis necessitates a co-ordination of left and right motor outputs, and for muscles like the diaphragm, where both sides must contract together, the activities of left and right phrenic nerves must be synchronised during the respiratory cycle. This synchronisation can be achieved by short-term synchronisation of the premotor neurone populations and by the excitation of left and right motoneurones from a common premotor neurone population. While the short-term synchronisation of left and right medullary premotor neurones has not been examined, cross-correlation analysis has detected the bilateral short-term synchronisation of left and right phrenic nerves due to their excitation by a common population of premotor neurones in adult rats (Duffin and van Alphen, 1995a; Tian and Duffin, 1996c). The transmission of such an excitation may proceed via three pathways: (1) premotor neurones with bifurcating axons projecting to each side of the neuraxis; (2) unilaterally projecting axons with a terminal arborisation crossing the midline; or (3) unilaterally projecting axons synapsing with midline-crossing motoneurone dendrites.

In adult rats, neuroanatomical tracing and electrophysiological studies show that the axons of phrenic premotor neurones bifurcate in the medulla to descend both left and right sides of the spinal cord (Dobbins and Feldman, 1994; Tian and Duffin, 1996c). A mid-sagittal transection of the medulla does not therefore interrupt the ipsilateral bulbospinal pathway (see figure 3.0), and left and right phrenic nerves retain their respiratory rhythm, albeit now asynchronously (see figure 3.2) (Peever et al., 1999b). From this observation, I suggest that bilateral short-term synchronisation of phrenic motoneurones does not result from either midline crossing terminal arborisation of premotor neurone axons or excitation of
midline-crossing motoneurone dendrites is ineffective. In adult rats, I therefore hypothesise that left and right phrenic motoneurones receive common synchronising inputs from premotor neurones with bifurcating axons projecting to each side of the neuraxis (see figure 4.0).

In neonatal rats, a functional examination of the short-term synchronisation of left and right phrenic nerve discharges has not been made in either intact or in-vitro neonatal rat preparations. While an anatomical tracing study examined projections to phrenic motoneurones from medullary bulbospinal neurones in 1 day-old rats, their left and right projections were not distinguished (Ellenberger, 1999). Based on mid-sagittal lesion studies in both intact neonatal rats (Janczewski and Aoki, 1997) and in-vitro brainstem-spinal cord preparations (McLean and Remmers, 1994), and from the observations presented in the previous chapter, I hypothesise that unlike adult rats, left and right phrenic motoneurones are not synchronised by bilaterally projecting phrenic premotor neurones. Rather, they are synchronised by bilaterally situated premotor neurones, which send axonal projections to the contralateral spinal cord (see figure 4.0).

With respect to the bilateral short-term synchronisation of hypoglossal nerve discharge very little is known; it has not been assessed using cross-correlation in either adults or neonatal rats. While the premotor neurones providing respiratory drive to hypoglossal motoneurones have been tentatively identified anatomically in the adult rat (Dobbins and Feldman, 1995) and cat (Ono et al., 1994), their projection pathways to left and right sides of the neuraxis are unknown. Nonetheless, I hypothesise a similar arrangement for hypoglossal premotor neurones as for phrenic premotor neurones (see figure 4.0).
Figure 4.0. A schematic representation of the hypothesised premotor pathways that transmit respiratory drive to hypoglossal (XII) and phrenic (C4) motoneurones in adult (A) and neonatal (B) rats. In A, XII and C4 premotor neurones have both ipsilateral and contralateral axons that project to both left and right sides, but in neonates (B) only the contralaterally projecting premotor neurons axons are established. Stars indicate functional projections.
With these considerations in mind, I carried out a series of experiments to assess the synchronisation of left and right hypoglossal and phrenic nerve discharges in both adult rats and in two neonatal in-vitro preparations; the brainstem-spinal cord and transverse brainstem-slice preparations. I used cross-correlation analysis of whole-nerve activities to detect short-term synchronisation of hypoglossal and phrenic motoneurones by excitation from a common premotor population.

The data presented below were presented as an abstract (Peever et al., 1999a) and an invited oral presentation at the 1999 Experimental Biology conference in Washington D.C., USA.

4.2 METHODS

4.2.1 Animal and Tissue Preparation

*In-vivo Preparations:* Adult male Sprague-Dawley rats weighing 350-500 grams were prepared by the methods described in Chapter 2, section 2.1.1.

*In-vitro Preparations:* Neonatal rats (2-3 day-old) were used to manufacture both in-vitro brainstem-spinal cord and transverse brainstem-slice preparations (see Chapter 2, section 2.1.3.).

4.2.2 Nerve Recording

Nerve were recorded as described in Chapter 2, section 2.3.1. An example of nerve recording is illustrated in figure 4.2.
Figure 4.1. A schematic showing the respiratory nuclei (VRG, ventral respiratory group) and respiratory motor nuclei (XII Mn, hypoglossal motor nuclei; C4 Mn, phrenic motor nuclei) with their associated nerves (XII, hypoglossal; C4, phrenic) in adult in-vivo and in-vitro brainstem-spinal cord preparations (A) and in in-vitro transverse brainstem slices (B). In A, the solid horizontal lines demonstrate where the brainstem-spinal cord of neonatal rats was sectioned to make in-vitro transverse brainstem slices.
4.2.3 Protocols

Cross-correlations of left and right phrenic and hypoglossal nerve discharges were computed in decerebrate adult rats and in in-vitro brainstem-spinal cord preparations (figure 4.1A), but in transverse brainstem-slice preparations only cross-correlations of left and right hypoglossal nerve rootlet discharges were computed (figure 4.1B). To test the ability of cross-correlation to detect common activation in the in-vitro preparations, the inspiratory discharges of two adjacent ipsilateral phrenic nerve rootlets were also cross-correlated (figure 4.1A). I did this because I thought that like adjacent intercostal nerves (Tian and Duffin, 1996b), adjacent phrenic motoneurones would be excited by a common population of inspiratory premotor neurones.

4.2.4 Data Analysis

Cross-correlation features were also described by their half-amplitude width; values are expressed as mean ± standard error, and statistical significance of features was considered at \( P < 0.01 \). Standard student \( t \)-tests were used to compare the half-amplitude width of cross-correlogram peaks. The level of confidence used was \( P < 0.05 \).

4.3 RESULTS

4.3.1 Bilateral Respiratory Discharge

All preparations exhibited a spontaneously rhythmic discharge on either left-right phrenic nerves or left-right hypoglossal nerves. A standard example of the rhythmic discharge recorded from bilateral hypoglossal nerve discharge is reported for adult (figure
and both the in-vitro brainstem-spinal cord (figure 4.2A) and transverse brainstem preparations (figure 4.2B).

4.3.2 Bilateral Short-term Synchronisation of Phrenic Nerve Discharge

In adult decerebrate rat preparations cross-correlograms computed between left and right phrenic nerve discharges (n = 4) displayed peaks at time zero, with a mean half-amplitude width of $1.4 \pm 0.1$ ms and mean bin counts of $4363 \pm 985$ (figure 4.3C, D). By contrast, those (n = 10) for in-vitro neonatal brainstem-spinal cord preparations (mean bin count $1936 \pm 449$) showed no statistically significant features (figure 4.4C, D).

The test of the cross-correlation analysis technique in the in-vitro preparations demonstrated its ability to detect common activation. Cross-correlograms (n = 6) computed between the discharges of adjacent ipsilateral phrenic nerve rootlets (mean bin count, $1171 \pm 328$) displayed statistically significant central peaks with a mean average half-amplitude width of $7.9 \pm 0.8$ ms (figure 4.5). The half-amplitude widths of these cross-correlograms are on average 5.6 times wider and significantly longer ($P = 0.001$) than those computed for left and right phrenic nerve activities in the adult (see figure 4.3C, D).

4.3.3 Bilateral Short-term Synchronisation of Hypoglossal Nerve Discharge

Cross-correlograms computed between left and right hypoglossal nerve discharges in adult rats (n = 12) displayed peaks at time zero with a mean half-amplitude width of $1.7 \pm 0.1$ ms and mean bin count of $6967 \pm 1337$ and (figure 4.3A, B). The mean half-amplitude width of these peaks is not significantly different ($P = 0.163$) from those computed for left and right phrenic nerves (see figure 4.3C, D). By contrast, cross-correlograms computed between
Figure 4.2. Typical inspiratory activities recorded from left and right hypoglossal (XII) nerves in brainstem-spinal cord in-vitro preparations (A), in transverse brainstem slice in-vitro preparations (B), and in adult in-vivo decerebrate rat preparations (C).
Figure 4.3. Representative cross-correlograms computed from the inspiratory activities of left and right hypoglossal (XII) nerves (A, B), and left and right phrenic (C4) nerves (C, D) in adult decerebrate rats. The central peaks were interpreted as evidence for synchronisation from a source of common excitation. Bin width is 0.2 ms.
Figure 4.4. Representative cross-correlograms computed from the inspiratory activities of left and right hypoglossal (XII) nerve rootlets (A, B), and from the inspiratory activities of left and right phrenic (C4) nerve rootlets (C, D) in brainstem-spinal cord preparations. The lack of features was interpreted as an absence of synchronisation from a source of common excitation. Bin width is 0.2 ms.
Figure 4.5. Cross-correlation histograms computed from the inspiratory activities of two ipsilateral phrenic (C4) nerve rootlets in the in-vitro brainstem-spinal cord preparation. The statistically significant broad central peaks were interpreted as evidence for synchronisation from a source of common excitation. Bin width is 0.2 ms.
left and right hypoglossal nerve rootlet discharges in *in-vitro* brainstem-spinal cord preparations (*n* = 10; mean bin count, 2528 ± 602) demonstrated no significant features (figure 4.4A, B). The cross-correlograms computed between left and right hypoglossal nerve discharges for transverse brainstem slices were also featureless (*n* = 6; mean bin count, 2071 ± 802), as shown in figure 4.6.

### 4.4 Discussion

From the cross-correlograms, I infer that left and right phrenic motoneurones are synchronously excited from a common premotor source in adults but not in neonates, as are hypoglossal motoneurones. I suggest that developmental changes occur in the transmission pathways to both phrenic and hypoglossal motoneurones in the rat with the contralateral pathways developing first and then the ipsilateral pathways (see below). Short-term synchronisation in the neonate therefore relies on a synchronisation of the premotor neurones while in the adult it is further strengthened by short-term synchronisation at the motoneurone level via bilaterally distributed pathways from premotor neurones. These observations are the first to document such *functional* changes in defined neuronal pathways of the central respiratory system occurring during development. My interpretations are discussed in detail below.

#### 4.4.1 Bilateral Short-term Synchronisation of Phrenic Nerve Discharge

In adult rats, cross-correlograms computed between left and right phrenic nerve discharges displayed central peaks. Duffin and van Alphen (1995a) report similar peaks (half-amplitude widths, ~1.8 ms), which they interpreted as evidence for both left and right
Figure 4.6. Cross-correlograms computed from the inspiratory activities of left and right hypoglossal (XII) nerve rootlets in the *in-vitro* transverse brainstem-slice preparation. The lack of features in cross-correlation histograms was interpreted as an absence of synchronisation from a source of common excitation. Bin width is 0.2 ms.
phrenic motor pools receiving excitation from a common inspiratory premotor neurone population. Indeed, inspiratory premotor neurones in the ventral respiratory group have axons that bifurcate to descend both left and right sides of the spinal cord (Dobbins and Feldman, 1994) and have been demonstrated to synaptically excite both left and right phrenic motoneurones in adult rats (Tian and Duffin, 1996b).

However, the short-term bilateral synchronisation detected by cross-correlation could also result if midline-crossing pathways via either premotor neurone terminal arborisation or motoneurone dendrite arborisation were effective. While the latter anatomical feature has been observed (Allan and Greer, 1997; Cameron et al., 1991a), it appears to be ineffective, at least in the neonatal rat and adult cat, because mid-sagittal transection of left and right phrenic motor nuclei has no discernible effect on phrenic nerve discharge (see Chapter 3, figure 3.3) (Eldridge and Paydarfar, 1989; Harada et al., 1985; Onimaru et al., 1989). Indeed, in the previous chapter, I showed that a midline transection of the adult rat brainstem resulted in independent rhythmic activities of left and right phrenic nerves with no evidence of bilateral synchronisation during the respiratory cycle. Therefore, I concluded that the bilateral short-term synchronisation of phrenic nerve discharges is solely due to excitation from a common population of medullary premotor neurones with bifurcating axons that descend both sides of the spinal cord.

In the in-vitro brainstem-spinal cord preparation, I observed no central peaks in cross-correlograms between right and left phrenic nerves, suggesting that they are not excited by a common premotor population. However, this observation could also have resulted if the cross-correlation technique was incapable of detecting common activation in these in-vitro preparations. The latter possibility is unlikely for two reasons. First, other investigators have
used cross-correlation analysis to detect short-term synchronous neuronal events in the \textit{in-vitro} brainstem-spinal cord preparation, and reported statistically significant features in cross-correlograms (Kashiwagi et al., 1993; Onimaru et al., 1993). Second, I found that cross-correlograms computed between adjacent phrenic nerve rootlets displayed central peaks. The cross-correlation technique was therefore capable of detecting a common activation and showed that a common population of inspiratory premotor neurones excites ipsilateral phrenic motoneurones. While this observation has not been confirmed for ipsilateral phrenic motoneurones, Tian and Duffin (1996b) report that in adult rats, cross-correlograms computed between the discharges of ipsilateral intercostal nerves display broad central peaks. They interpret these peaks to indicate that the ipsilateral motoneurones of adjacent motor pools receive a common source of premotor excitation.

The half-amplitude widths of central peaks generated between ipsilateral phrenic nerve rootlets were approximately 5.6 times wider than those computed between the whole-nerve discharges of left-right phrenic nerves in adult rats. Similarly, Kashiwagi et al. (1993) report that very broad central peaks were detected in cross-correlograms computed between contralateral ventral respiratory group inspiratory neurones in \textit{in-vitro} brainstem-spinal cord preparations. I suggest, like Kashiwagi et al. (1993), that this characteristic was due to slower transmission times because of the reduced temperature (Hille, 1984) and immature myelination of axons (Fitzgerald, 1985) of \textit{in-vitro} preparations. Nonetheless, I conclude that the cross-correlation technique is fully capable of detecting common synchronising inputs between nerve rootlets in these \textit{in-vitro} preparations.

I postulate that the differences between the bilateral synchronisation detected in the adult and its absence in the neonatal \textit{in-vitro} preparations might be due to immaturity in the
transmission pathway in the latter. Specifically, I suggest that it is the ipsilateral bulbospinal pathway that is not functionally established in the neonatal in-vitro preparations. This contention is supported by the mid-sagittal transection observations reported in Chapter 3. I showed that mid-sagittal transection of the adult medulla desynchronises the left and right phrenic nerve discharges (see figure 3.2) (Peever et al., 1998); and interpret this observation to indicate that the contralateral bulbospinal pathway is severed leaving the ipsilateral pathway intact to convey independent respiratory rhythms (figure 3.0 and 4.0). Similarly, Janczewski and Aoki (1997) found that mid-sagittal transection of the medulla abolished respiratory motor output in 2-9 day-old anaesthetised neonatal rats, the same age as my preparations, but not in 17-22 day-old rats. I interpret these observations to indicate that, as in the adult, mid-sagittal medullary transection severs the contralateral bulbospinal pathway, but in this case, phrenic nerve output fails because the ipsilateral bulbospinal pathway is not functionally developed in the 2-9 day-old rats. Transection findings also demonstrate that in the neonatal in-vitro preparation the contralateral branch crosses the midline rostral to the most caudal hypoglossal nerve rootlets, because transections caudal to this level do not eliminate phrenic nerve discharge (see Chapter 3).

4.4.2 Bilateral Short-term Synchronisation of Hypoglossal Nerve Discharge

In adult rats, cross-correlograms computed between left and right hypoglossal nerve discharges displayed central peaks, whereas those for neonatal in-vitro preparations did not. I hypothesise that like phrenic nerves, left-right hypoglossal nerves receive excitation from a common population of medullary premotor neurones. Alternatively, a common excitation may also be conveyed via unilaterally projecting axons with a terminal arborisation crossing
the midline, or via unilaterally projecting axons synapsing with midline-crossing motoneurone dendrites. These possibilities cannot be distinguished using the mid-sagittal transection reported in the previous chapter (as for the phrenic nerves), because midline transection not only severs possible axonal transmission pathways but also the midline-crossing pathways of rhythm-generating neurones themselves. Although the location of hypoglossal premotor neurones have been tentatively identified in adult cat and rat (Dobbins and Feldman, 1995; Ono et al., 1994) whether they possess bilaterally bifurcating axons like phrenic premotor neurones is unknown. According to Ono et al. (1994, 1998), hypoglossal and phrenic motoneurones are excited by a common population of premotor neurones in adult cats. I contend that in adult rats, it is also possible for a common population of premotor neurones to synchronously excite both hypoglossal and phrenic motoneurones, and will test this hypothesis in the following chapter. In addition, in adult rats, neuroanatomical studies demonstrate that hypoglossal motoneurones possess midline-crossing motoneurone dendrites (Aldes, 1995; Núñez-Abades et al., 1994), and so this remains a possible transmission pathway. However, I suspect that left and right hypoglossal nerves are bilaterally synchronised in the same manner that left and right phrenic nerve are, that is, via a common population of premotor neurones with bilateral axonal projections to left and right sides of the medulla. This hypothesis will be examined in Chapter 6.

In the in-vitro brainstem-spinal cord and transverse brainstem-slice preparations, I observed no central peaks in cross-correlograms between right and left hypoglossal nerves, suggesting that a common premotor population does not excite them. Anatomical examination of hypoglossal motoneurones in the neonate shows that, as in the adult, they possess midline-crossing dendrites (Aldes, 1995; Mazza et al., 1992; Núñez-Abades et al.,
1994). The lack of bilateral short-term synchronisation by excitation from a common premotor population therefore indicates that these midline-crossing motoneurone dendrites are not an effective transmission pathway in the neonatal *in-vitro* preparation. Synchronisation pathways are therefore either via bifurcating axons or midline-crossing axonal terminal arborisations.

Nevertheless, I suggest that the synchronisation pathway in the neonatal *in-vitro* preparations is via contralateral bifurcating axons rather than midline-crossing terminal arborisation, because mid-sagittal transection at the level of the rostral hypoglossal nerve rootlets abolishes hypoglossal nerve discharge. Thus, although both of the possible synchronising pathways are severed, the cessation of discharge shows that the transmission pathway cannot be via ipsilateral axons (which would not be severed) with midline-crossing terminal arborisation (which would be severed) because then the respiratory nerve discharge should not fail in that case. I conclude that like phrenic motoneurones, the bilateral synchronisation of left-right hypoglossal motoneurones arises from the bifurcating axons of contralateral premotor neurones.

One other interpretation of the midline transection findings must be considered; it is possible that the mid-sagittal transection disrupts the generation of respiratory rhythm in the neonatal *in-vitro* preparations. However, as I argued in Chapter 3, section 3.4.1, the recent demonstration by Johnson et al. (2000) that islands containing the pre-Bötzing complex isolated from transverse slices can generate rhythm, show that this alternative is untenable.

If bifurcating axons of hypoglossal premotor neurones are responsible for short-term bilateral synchronisation in the same way as for phrenic motoneurones, then my cross-correlograms suggest that contralateral projections develop before ipsilateral projections. As
for the phrenic nerve, I suggest that the differences between the bilateral short-term synchronisation detected in the adult and its lack in the neonatal *in-vitro* preparations might be due to immaturity in the transmission pathway in the latter. Specifically, I suggest that it is the ipsilateral pathway that is not functionally established in the neonatal *in-vitro* preparations, because mid-sagittal transection at the level of the rostral hypoglossal rootlets eliminates nerve discharge.

### 4.4.3 Development of Respiratory Rhythm Transmission

That the medullary respiratory control system is subject to developmental change seems reasonable (Bianchi et al., 1995; Duffin et al., 1995; Hilaire and Duron, 1999; St.-John, 1998). During postnatal development, the neuronal properties of the mammalian brain change, and neuronal pathways undergo reorganisation and redistribution (Christensen et al., 1999; Rinaman et al., 2000; Fitzgerald and Jennings, 1999; Glover, 2000; Theriault and Tatton, 1989).

In support of this view, the pattern of respiratory motor nerve discharge has been observed to change during development (Ramirez et al., 1996; Smith et al., 1990), either because of alterations in the electrophysiological properties of the respiratory motoneurones themselves or from changes in interconnexions within the brainstem respiratory network. The former hypothesis is supported by a number of investigations (Cameron et al., 1991a; Martin-Caraballo and Greer, 1999; Viana et al., 1994), including those that observed intercellular electrical coupling in both cranial and spinal respiratory motoneurone populations in neonates but not in adults (Allan and Greer, 1997; Mazza et al., 1992; Rekling and Feldman, 1997; van der Want et al., 1998). The latter hypothesis, that developmental
changes may occur in the medullary respiratory network connexions, is supported by the finding that synaptic inhibition is required for the generation of respiratory rhythm in adult rats (Hayashi and Lipski, 1992), but in neonatal rodents it is not (Ramirez et al., 1996; Shao and Feldman, 1997).

Support for developmental changes in the pathways transmitting respiratory rhythm to motoneurones originates in observations such as those by Ramirez et al. (1996). They found changes in the coupling of bursting activity from ventral respiratory group inspiratory neurones to hypoglossal nerves with development. In 0–4 day-old mice each burst in the hypoglossal nerve rootlet corresponded to one burst of ventral respiratory group inspiratory neurones, but in 5–18 day-old mice one burst in the hypoglossal nerve rootlet occurred every third or fourth burst in ventral respiratory group inspiratory neurones.

Although there is no direct neuroanatomical evidence to demonstrate that respiratory transmission pathways undergo functional reorganisation during postnatal development, there is accumulating evidence that the premotor pathways of other motor systems change during early development. Generally, neuronal connexions crossing the midline are more substantial in the immature spinal cord (Lowrie, 1999), and this is evidenced by neuroanatomical studies. In both the developing cat and monkey, axonal projections of bilateral corticospinal premotor neurones are denser contralaterally than ipsilaterally, but during the first few weeks to months of postnatal development the ipsilateral projections become denser (Galea and Darian-Smith, 1995; Martin and Lee, 1999; Martin et al., 1999). A similar explanation might be applied to respiratory developing transmission pathways in the neonatal rat.
4.4.4 Comparison of *In-vivo* and *In-vitro* Preparations

Based on the observations detailed in Chapter 1, section 1.6.4, it could be argued that the rhythm generated in the reduced *in-vitro* preparations is not eupnoeic in character but corresponds to gasping. It is therefore plausible that there are separate transmission pathways for each mode of rhythm generation; in which case, the rhythm transmission pathways in my *in-vitro* preparations are not appropriate models of the intact neonate and cannot be compared with those of adults to deduce developmental changes. While I cannot rule out this possibility, I do not consider it likely for the reasons detailed in Chapter 1 and 3, sections 1.6.4 and 3.4.3, respectively. Rather, I suggest that my findings indicate that developmental changes occur in the transmission of respiratory rhythm to phrenic and hypoglossal motoneurones.

4.4.5 Conclusions

The cross-correlation findings show that left and right phrenic and left and right hypoglossal motoneurones are synchronously excited from common premotor neurone populations in adult rats but not in neonatal rat *in-vitro* preparations. When considered in concert with results from the mid-sagittal transection experiments in Chapter 3, these findings demonstrate that respiratory rhythm is transmitted to both phrenic and hypoglossal motoneurones via premotor neurones with bilateral projections in the adult, but with unilateral contralateral projections in *in-vitro* preparations. I speculate that respiratory transmission pathways undergo developmental changes and suggest that contralateral connexions develop before the ipsilateral ones.
CHAPTER 5

RESPIRATORY CONTROL OF HYPOGLOSSAL MOTONEURONES

5.0 SUMMARY

In adult and neonatal rats, I used cross-correlation analysis to detect synchronous neuronal events in hypoglossal and phrenic nerves, to infer synaptic connexions. I determined whether: (1) motoneurones that comprise the medial and lateral hypoglossal nerve branches receive inspiratory excitation from the same inspiratory premotor population; (2) the inspiratory premotor population exciting hypoglossal motoneurones is the same as that exciting phrenic motoneurones; and (3) hypoglossal motoneurones are inhibited by Bötzinger complex expiratory neurones as are phrenic motoneurones. In 12 anaesthetised adult rats, I computed cross-correlograms between the inspiratory activities of medial and lateral hypoglossal nerve branches. They displayed narrow peaks at the origin (mean half-amplitude width, 0.5 ± 0.1 ms), indicative of common excitation. In 6 in-vitro brainstem-spinal cord preparations from neonatal rats, cross-correlograms computed between adjacent hypoglossal nerves were featureless. In adult and neonatal rat preparations, I computed cross-correlograms between the inspiratory activities of phrenic and hypoglossal nerves. In 23 adult and 10 neonatal rat preparations, the cross-correlograms were featureless; no common activation was evident. To confirm this negative result, I extracellularly recorded the activity of inspiratory neurones in the ventral respiratory group, identified them as phrenic premotor neurones, and computed their cross-correlograms with the activity of the hypoglossal nerves. In 23 adult rats, 18 ipsilateral and 8 contralateral inspiratory neurones

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were identified as premotor to phrenic motoneurones from cross-correlogram peaks (half-amplitude width, 1.27 ± 0.09 ms; latency, 1.77 ± 0.15 ms). However, only 1 of the 26 displayed a cross-correlogram with a peak (half-amplitude width, 0.6 ms; latency, 1.6 ms) indicating an excitation of hypoglossal motoneurones. I activated the axons of inhibitory Bötzinger complex expiratory neurones with stimuli (0.2 ms pulses, 10-25 μA, 5 Hz) applied via glass-coated tungsten microelectrodes placed into the lateral spinal cord at the border of the second and third cervical segments. In 15 adult rats, post-stimulus histograms for the phrenic nerve displayed narrow troughs (half-amplitude widths, 2.52 – 2.66 ms) but were featureless for the hypoglossal nerve. From these studies, I conclude that: (1) in adult rats, motoneurones of the medial and lateral hypoglossal nerve branches receive inspiratory drive from a common premotor population, but in neonatal rats they do not; (2) in both adult and neonatal rats, hypoglossal motoneurones are not monosynaptically excited by the inspiratory neurones that are premotor to phrenic motoneurones; (3) hypoglossal motoneurones are not inhibited by the Bötzinger complex expiratory neurones that inhibit phrenic motoneurones. I therefore suggest that respiratory control of hypoglossal motoneurones is separate from that for phrenic motoneurones.

5.1 INTRODUCTION

The tongue is an important pharyngeal muscle that participates in a variety of behaviours. In both adult and newborn mammals it is used for phonation, swallowing, chewing, lapping, drinking, suckling, coughing, vomiting and breathing (Lowe, 1981). During breathing the position of the tongue is important to ensure airway patency. Tongue position is controlled primarily by the genioglossus muscle (tongue protruder), hyoglossus
and styloglossus muscles (tongue retractors) and intrinsic muscles. The tongue protrudor and retractor muscles are innervated by hypoglossal motoneurones, which are somatotopically organised in bilateral nuclei extending along the midline of the medulla (Dobbins and Feldman, 1995; Uemura-Sumi et al., 1988; Uemura-Sumi et al., 1981). The hypoglossal nerve bifurcates to form medial and lateral branches; the medial branch innervates the protrusor tongue muscles, and the lateral branch innervates the retractor tongue muscles (Barnard, 1940; Dobbins and Feldman, 1995; Lewis et al., 1971; Uemura-Sumi et al., 1988; Uemura-Sumi et al., 1981). During the inspiratory phase of the respiratory cycle, tongue protrusor and retractor muscles are simultaneously activated (Fregosi and Fuller, 1997) to stiffen tongue muscles and thereby maintain a patent pharyngeal airway; allowing the diaphragm and other inspiratory pressure-generating muscles to draw air into the lungs (Brouillette and Thach, 1980; Fregosi and Fuller, 1997; Remmers et al., 1978). Thus, the rhythmic contractions of the upper airway and chest wall muscles, which are controlled by a network of neurones located within the medulla and pons (Bianchi et al., 1995; Duffin et al., 1995; St.-John, 1998), must occur in an appropriate sequence. However, the premotor circuitry that is responsible for this finely controlled sequence is not well understood. To this end, I examined respiratory premotor control of hypoglossal and phrenic motoneurones in both adult and neonatal populations.

In rats, both medial and lateral hypoglossal nerve branches and phrenic nerves exhibit bursts of activity coincident with inspiration and are quiescent during expiration. The pathways by which respiratory drive is transmitted from the medullary respiratory rhythm-generating network to phrenic motoneurones are well characterised both anatomically and electrophysiologically (Lipski et al., 1994; Ono et al., 1994; Tian et al., 1998c). Bulbospinal
inspiratory neurones of the rostral ventral respiratory group transmit inspiratory drive to phrenic motoneurones (Liu et al., 1990; Monteau and Hilaire, 1991; Tian and Duffin, 1996b) and bulbospinal expiratory neurones in the Bötzinger complex actively inhibit these motoneurones during expiration (Tian et al., 1998a). However, functional identification of the premotor pathways transmitting respiratory drive from the rhythm-generating network to hypoglossal motoneurones is lacking.

Stimulation of the medial hypoglossal nerve branch protrudes the tongue, whereas stimulation of the lateral nerve branch retracts it (Eisele et al., 1997; Lewis et al., 1971). During normal breathing, some studies report that tongue protrusor muscles are phasically active during inspiration (Andrew, 1955), whereas the retractors are not (Doty and Bosma, 1956). These functional differences between retractor and protrusor muscles led to the suggestion that separate premotor neurone populations transmit respiratory drive to protrusor and retractor hypoglossal motoneurones. Putative hypoglossal premotor neurones are located in the lateral tegmental field (Dobbins and Feldman, 1995; Fenik et al., 1998; Ono et al., 1994; Ono et al., 1998), and the neuroanatomical findings of Dobbins and Feldman (1995) in rats support a separation of premotor populations. However, findings from recent studies have demonstrated that both protrusor and retractor tongue muscles are co-activated during inspiration. This finding implies that motoneurones innervating these muscles must also be synchronously excited, and I therefore hypothesise that a common population of inspiratory premotor neurones transmit inspiratory drive to medial (protrusor) and lateral (retractor) hypoglossal motoneurones (see figure 5.0). To test this hypothesis, I cross-correlated the whole-nerve activities of medial and lateral hypoglossal nerve branches in anaesthetised
Figure 5.0. A schematic representation of the hypothesised premotor pathways that transmit respiratory drive to hypoglossal (XII) and phrenic (C4) motoneurones. In A, I hypothesise that inspiratory neurons within the ventral respiratory group (VRG-Inspiratory Neurons) transmit inspiratory drive to both C4 and medial and lateral XII motoneurones. In B, I hypothesise that Bötzinger complex expiratory neurones (Böt-Expiratory Neurones) synaptically inhibit both C4 and XII motoneurones. The symbols, + and -, indicate excitatory and inhibitory connexions, respectively.
adult rats, and adjacent hypoglossal nerve rootlets in neonatal rat in-vitro isolated brainstem-spinal cord preparations to detect common excitation (see figure 5.1).

Some investigators suggest that in both adult cats and newborn rats, phrenic and hypoglossal motoneurones receive respiratory drive from different inspiratory premotor populations (Morin et al., 1992; Sica et al., 1984). However, Ono et al. (1994, 1998) suggest that in cats, a population of medullary inspiratory premotor neurones located dorsomedial to the nucleus ambiguous transmits a common respiratory drive to both phrenic and hypoglossal motoneurones (see figure 5.0). To test this hypothesis two series of experiments were performed in adult and neonatal rat preparations. First, I cross-correlated the inspiratory activity of the phrenic and hypoglossal nerves to detect common synchronising inspiratory inputs. Then, I simultaneously cross-correlated the extracellular activity of putative inspiratory phrenic premotor neurones with the inspiratory activity of phrenic and hypoglossal nerves in adult decerebrate rats, to determine if those inspiratory neurones, premotor to phrenic motoneurones, were also premotor to hypoglossal motoneurones.

Hypoglossal motoneurones are inhibited by vagus and superior laryngeal nerve afferent stimulation (Fukuda and Honda, 1982b; Ono et al., 1998; Sica et al., 1984); however, it is unknown whether rhythm-generating neurones themselves modulate the activity of hypoglossal motoneurones during expiration. Bötzinger complex expiratory neurones are involved in the generation and modulation of respiratory rhythm in adult mammals (Duffin et al., 1995), and inhibit different groups of respiratory neurones (Bianchi et al., 1995), including phrenic motoneurones (Tian et al., 1998a). I postulated that, like phrenic motoneurones, hypoglossal motoneurones are actively inhibited by the expiratory neurones of the Bötzinger complex (see figure 5.0). To test this hypothesis in adult rats, I stimulated
Figure 5.1. A schematic representation of the respiratory nuclei, respiratory motoneurones, and nerves contained within the brainstem and spinal cord of the rat recorded from both adult (left) and neonatal (right) preparations. The ventral respiratory group (VRG), the putative rhythm-generator; hypoglossal motoneurones (XII Mn), which supply both medial and lateral hypoglossal (XII) nerves; and phrenic motoneurones (C4 Mn)., which supply the phrenic (C4) nerves; and Bötzinger complex expiratory neurones (Böt), illustrate the hypothesized premotor neurones innervating XII and C4 Mn.
the axons of Bötzinger complex expiratory neurones in the spinal cord while simultaneously computing post-stimulus histograms of both phrenic and hypoglossal nerve activities to detect whether both were inhibited.

These experiments were therefore designed to determine whether motoneurones of the medial and lateral hypoglossal nerve branches receive inspiratory excitation from a common premotor source and to establish whether the inspiratory and expiratory premotor neurones that transmit respiratory rhythm to phrenic motoneurones also transmit it to hypoglossal motoneurones (see figure 5.0). I conclude that medial and lateral branches of the hypoglossal nerve receive inspiratory excitation from a common premotor population, and that inspiratory and expiratory modulation of hypoglossal motoneurones is not mediated by inspiratory and expiratory phrenic premotor neurones. It is my contention that respiratory control of hypoglossal motoneurones is separate from that for phrenic motoneurones.

This work has been presented as an abstract (Peever and Duffin, 2000), and has been accepted for publication by Pflügers Archives-European Journal of Physiology.

5.2 METHODS

5.2.1 Animal and Tissue Preparation

I used in-vivo, decerebrate and anaesthetised adult rat preparations as models of the mature respiratory system (see Chapter 2 sections 2.1.1. and 2.1.2), and in-vitro brainstem-spinal cord preparations isolated from neonatal (1-5 day old) rats as a model of the immature respiratory system (see Chapter 2, section 2.1.3).
5.2.2 Nerve and Cellular Recordings

Nerves and neurones were recorded as described in Chapter 2, section 2.3.1. Examples of recordings are illustrated in figure 5.2.

5.2.3 Protocols

Figure 5.1 is a detailed schematic depiction of recording procedures in both adult and in-vitro neonatal rat preparations. In adult rats, I cross-correlated the inspiratory activities of: (1) hypoglossal and phrenic nerves (decerebrate rats); and (2) medial and lateral branches of the hypoglossal nerve (anaesthetised rats). In decerebrate rats, extracellular searches were made for inspiratory ventral respiratory group neurones and their activities were cross-correlated with the inspiratory activities of the hypoglossal and phrenic nerves. In adult rats, the axonal tracts of Bötzinger complex expiratory bulbospinal neurones are located at the border of the second and third cervical segments of the spinal cord (Douse and Duffin, 1992; Tian et al., 1998a); a stimulating microelectrode was positioned at this level to anti- and orthodromically activate Bötzinger complex expiratory neurones while post-stimulus histograms were computed with the inspiratory activities of phrenic and hypoglossal nerves. In in-vitro brainstem-spinal cord preparations, I cross-correlated the inspiratory activities of: (1) phrenic and hypoglossal nerve rootlets; (2) adjacent hypoglossal nerve rootlets; and (3) adjacent phrenic nerve rootlets.
Figure 5.2. **Adult:** Examples of the inspiratory discharge of an ipsilateral phrenic nerve (top trace, C4), a ventral respiratory group inspiratory neurone (middle trace, VRG-I), and a hypoglossal nerve (bottom trace, XII) recorded from an adult, decerebrate rat. **Neonatal:** Examples of the inspiratory discharge recorded from ipsilateral hypoglossal (top trace, XII) and phrenic (lower trace, C4) nerve rootlets of an *in-vitro* brainstem-spinal cord preparation from a 4 day-old rat.
5.3 RESULTS

5.3.1 Cross-Correlation Analysis of Hypoglossal Nerves

5.3.1.1 Anaesthetised Adult Rats

The onset times for the inspiratory discharge of both medial and lateral hypoglossal nerves were identical. Cross-correlograms computed between medial and lateral branches of the hypoglossal nerve discharges \( (n = 12) \) displayed narrow statistically significant peaks at time zero with a mean half-amplitude width of \( 0.5 \pm 0.1 \) ms and a mean bin count of \( 759 \pm 484 \) (figure 5.3).

5.3.1.2 In-Vitro Brainstem-Spinal Cord Preparations

The onset times for the inspiratory discharge were identical, but in 1 of the 6 preparations one hypoglossal nerve rootlet discharged in advance of the other by \( \sim 20 \) ms. Cross-correlograms computed between adjacent hypoglossal nerve rootlet discharges in brainstem-spinal cord preparations were featureless (mean bin count, \( 3179 \pm 589 \)), as shown in figure 5.4 A, B.

To validate the cross-correlation analysis technique in the in-vitro preparation, I cross-correlated the inspiratory activity of two adjacent ipsilateral phrenic (fourth cervical rootlet) nerve rootlets \( (n = 6) \). Cross-correlograms (mean bin count, \( 1171 \pm 328 \)) revealed statistically significant central peaks with an average half-amplitude width of \( 7.9 \pm 0.8 \) ms (figure 5.4 C, D).
Figure 5.3. Two examples of cross-correlograms computed between the inspiratory activities of medial and lateral hypoglossal (XII) nerve branches in anaesthetised adult rats showing central peaks indicative of common activation. Bin widths are 0.2 ms.
Figure 5.4. Examples of cross-correlograms computed between the inspiratory activity of adjacent ipsilateral hypoglossal (XII) nerve rootlets in *in-vitro* brainstem-spinal cord preparations from 2 day-old (A) and 4 day-old rats (B) showing no significant features, and between the inspiratory activities of adjacent ipsilateral phrenic (C4) nerve rootlets in *in-vitro* brainstem-spinal cord preparations from 3 day-old rats (C, D) showing central broad peaks indicative of common activation. Bin widths are 0.2 ms.
5.3.2 Cross-Correlation Analysis of Hypoglossal and Phrenic Nerves

5.3.2.1 Decerebrate Adult Rats

In 23 adult rats, hypoglossal and phrenic nerves discharged during inspiration, but not in synchrony (figure 5.2 and 5.5, top traces); in all cases, hypoglossal nerves discharged in advance of phrenic nerves by 180 ± 7 ms (figure 5.5). Cross-correlation histograms (figure 5.6 A, B) computed between the hypoglossal nerve and the ipsilateral phrenic nerve were featureless (mean bin count, 9799 ± 1661) as were those computed between the hypoglossal nerve and the contralateral phrenic nerve in 6 rats (mean bin count, 5893 ± 1470).

5.3.2.2 In-Vitro Brainstem-Spinal Cord Preparations

In 10 preparations phrenic and hypoglossal nerve rootlets both exhibited inspiratory discharge (figure 5.2 and 5.5, bottom traces). However, in 8 of the 10 preparations, the onset of phrenic nerve discharge preceded the hypoglossal nerve by 60 ± 5 ms (figure 5.5); in the remaining 2 preparations the onset of inspiratory discharge was the same in both nerves. Cross-correlation histograms (figure 5.6 C, D) computed between the ipsilateral phrenic and hypoglossal were featureless (mean bin count, 3406 ± 479) as were those computed between contralateral hypoglossal and phrenic nerves in 6 rats (mean bin count, 2677 ± 1069).

5.3.3 Cross-Correlation Analysis of Inspiratory Neurones and Hypoglossal/Phrenic Nerves

Extracellular recordings were made from 26 inspiratory neurones. Figure 5.7 shows their locations with respect to the obex, determined from micromanipulator coordinates; 18 were located in the ipsilateral ventral respiratory group and 8 in the contralateral ventral respiratory group. All neurones were identified as premotor to phrenic motoneurones from
Figure 5.5. Examples of the inspiratory activities of hypoglossal (XII) (top traces) and phrenic (C4) (bottom traces) nerves in both adult (A) and neonatal in-vitro (B) rat preparations illustrating the disparity of onset times of XII and C4 nerves.
Figure 5.6. Examples of cross-correlograms computed between the inspiratory activities of phrenic (C4) and hypoglossal (XII) nerves in adult (top) decerebrate rats (A, B) and in in-vitro brainstem-spinal cord preparations (bottom) from 2 day-old (C) and 3 day-old (D) rats. Bin widths are 0.2 ms.
Figure 5.7. Microelectrode co-ordinates from micromanipulator readings relative to the obex for the ventral respiratory group inspiratory neurones recorded in adult decerebrate rats. Top, sagittal view; middle, dorsal view; and bottom, transverse view.
cross-correlation histogram peaks (1.27 ± 0.09 ms mean half-amplitude width) at short latencies (1.77 ± 0.15 ms) as shown in figure 5.8, right side. Of the 26 phrenic premotor neurones, the cross-correlogram with hypoglossal nerve discharge for one ipsilateral neurone had narrow peak (half-amplitude width = 0.6 ms) with a latency of 1.6 ms (figure 5.8 top left); the rest were featureless (figure 5.8, left side).

5.3.4 Post-Stimulus Histograms for Phrenic and Hypoglossal Nerves

In 15 decerebrate adult rats, I recorded the activities of phrenic and hypoglossal nerves and stimulated (0.2 ms pulses, 10-25 μA, 5 Hz) the axons of Böttinger complex expiratory neurones (see figure 5.1). Post-stimulus histograms with hypoglossal nerves revealed no features (figure 5.9, A2, B2, C2), but showed one of the following arrangements of features with phrenic nerves. (1) A trough (mean half-amplitude width, 2.52 ± 0.73 ms) at a short latency (3.78 ± 0.79 ms) for 5 (figure 5.9 A1). (2) A trough (mean half-amplitude width, 2.66 ± 0.61 ms) at a short latency (2.66 ± 0.22 ms) accompanied by a peak (mean half amplitude width, 2.60 ± 0.52 ms) at a longer latency (7.50 ± 0.74 ms) for 5 (figure 5.9 B1). (3) A narrow peak (half-amplitude widths, 0.94 ± 0.44 ms) at a short latency (2.78 ± 0.18 ms) followed by a broad trough (mean half amplitude width, 3.30 ± 0.40 ms) at a longer latency (5.02 ± 0.54 ms) for 5 (figure 5.9 C1).

5.4 DISCUSSION

Based on these results, I propose that motoneurones of the medial and lateral hypoglossal nerve branches receive inspiratory drive from a common premotor population in adult rats, but in neonatal rats they do not, from which I surmise that developmental changes occur. Further, I suggest that respiratory control of hypoglossal motoneurones is separate
Figure 5.8. Cross-correlograms computed between the inspiratory activities of ventral respiratory group inspiratory neurones and hypoglossal and phrenic nerves in decerebrate adult rats. Bin widths are 0.2 ms and arrows point to statistically significant peaks.
Figure 5.9. Post-stimulus histograms for phrenic (left) and hypoglossal (right) nerves in decerebrate, adult rats illustrating the three types of responses observed. Stimulus artefacts are visible at ~ 0.6 ms. Bin widths are 0.2 ms and arrows point to statistically significant features.
from that for phrenic motoneurones because in both adult and neonatal rats, hypoglossal motoneurones are not excited by the inspiratory neurones that are premotor to phrenic motoneurones; neither are hypoglossal motoneurones inhibited by the Bötzing her expiratory neurones that inhibit phrenic motoneurones. The functional significance of these findings is discussed after consideration of my interpretations.

5.4.1 General Considerations

In these experiments, I investigated the sources of respiratory drives to hypoglossal motoneurones in both adult and neonatal rats. I used well-established adult and in-vitro neonatal preparations. Although the adult rat preparations retain their afferent inputs, whereas the in-vitro brainstem-spinal cord preparations do not, I believe the essential neuronal circuitry responsible for the generation and transmission of respiratory rhythm to be comparable between them (see Chapter 1, 3 and 4) (Peever et al., 1999b), as do other investigators (Smith et al., 1990; Suzue, 1984). Certainly the in-vitro preparation is relatively hypoxic and hypercapnic (Okada et al., 1993) and at a lower temperature (26-29°C) than intact neonates.

Despite differences between intact and in-vitro preparations, they spontaneously generate periodic bursts of neural activity on phrenic and hypoglossal nerve rootlets (Smith et al., 1990; Suzue, 1984). In addition, other investigators have observed respiratory neurones with firing patterns similar to those of the ventral respiratory group in adult rats; including pre-inspiratory, inspiratory, expiratory and phase-spanning neurones (Onimaru and Homma, 1992; Onimaru et al., 1992; Smith et al., 1990). Furthermore, I demonstrated that when the temperature of the in-vitro brainstem-spinal cord preparation is increased to within
physiological limits (35°C), the frequency and pattern of phrenic motor output approaches that of intact age-matched neonatal rats (Peever et al., 1999b). The in-vitro brainstem-spinal cord preparation is therefore a useful model of the central respiratory system to compare with the adult rat for deducing developmental differences.

5.4.2 Relationship of Ipsilateral Hypoglossal Nerve Activities

Because it has been recently demonstrated in adult rats that the protrusor and retractor tongue muscles are co-activated during normal breathing (Fregosi and Fuller, 1997), I hypothesized that both hypoglossal nerve branches would receive a common excitation during inspiration. However, Dobbins and Feldman (1995) reported anatomical evidence for a differential premotor projection to hypoglossal motoneurones; with premotor neurones driving motoneurones in the medial nerve branch situated more ventral and ventromedial within the lateral tegmental field than those driving motoneurones in the lateral nerve branch.

In my experiments, I observed that both medial and lateral hypoglossal nerve branches discharge in synchrony with one another, and that cross-correlograms computed between the inspiratory discharge of these nerves reveal narrow peaks at time zero. Taken together these functional observations strongly suggest that the motoneurones of the medial and lateral hypoglossal nerve branches receive inspiratory excitation from a common population of inspiratory premotor neurones. This finding is supported by recent experiments showing co-activation of medial and lateral hypoglossal nerves when either upper airway pressure decreases (Fuller et al., 1999; Ryan et al., 2000) or respiratory drive increases (Fuller et al., 1998).
How tongue protrusor and retractor muscles participate in normal breathing in neonatal rats is unknown, although it is generally assumed they serve the same functions and are controlled by similar premotor circuits as in adult rats (Ballanyi et al., 1999). Here, I present evidence that premotor control of hypoglossal motoneurones differs between adult and neonatal rats. In neonatal rats I found no evidence in support of the hypothesis that motoneurones comprising adjacent hypoglossal nerve rootlets receive inspiratory drive from a common premotor population. Possible methodological explanations for this finding include differences between the in-vivo and in-vitro preparations, and differences in nerve recording methods and the sensitivity of the cross-correlation technique. The first explanation was discussed above (see General Considerations) and detailed in Chapter 1, section 1.6.4; I believe that it is not the explanation. To address the second possibility I cross-correlated the discharges recorded from adjacent phrenic nerve rootlets; the cross-correlograms displayed central peaks, thereby demonstrating that the method was indeed sensitive enough to detect common activation. Similarly, other investigators have used cross-correlation analysis to detect synchronous neuronal events in the in-vitro brainstem-spinal cord preparation, and reported statistically significant features in cross-correlograms (Kashiwagi et al., 1993; Onimaru et al., 1993). The peaks are wider in these preparations than in adults because of the slower transmission time, which is due to the reduced temperature and immature myelination of axons in the neonatal central nervous system (Fitzgerald, 1985; Kashiwagi et al., 1993).

One further objection may be raised; the differences could be due to the fact that I recorded from medial and lateral hypoglossal nerve branches in adult rats and from adjacent hypoglossal nerve rootlets in neonatal preparations. However, I believe this is not the reason.
Indeed, I argue that testing adjacent rootlets for shared excitation in the neonate is an even more rigorous test than testing for shared excitation of medial and lateral XII nerves in the adult. Due to the somatotopic organisation of the hypoglossal motor nucleus (Dobbins and Feldman, 1995; Uemura-Sumi et al., 1988; Uemura-Sumi et al., 1981) adjacent nerve rootlets should originate from motoneurones in close proximity, and these are likely to share synaptic inputs from the same source even if respiratory premotor neurones have only a limited terminal arborisation in the motor nucleus.

Furthermore, phrenic and hypoglossal motoneurones are functionally mature in neonatal in-vitro brainstem-spinal cord preparations, and their intrinsic membrane properties are similar (Morin et al., 1992). Therefore, differences between adult and neonatal preparations are unlikely to be due to functionally immature hypoglossal motoneurones in the neonate. This conclusion is indirectly supported by my finding of a common activation for adjacent phrenic nerve rootlets but not for adjacent hypoglossal nerve rootlets. Since I demonstrated that adjacent hypoglossal motoneurones receive inspiratory excitation from a common premotor population in adults, the lack of such short-term synchronisation in the neonatal preparation suggests that the terminal arborisations of premotor neurones among hypoglossal motoneurones is inadequate. I surmise that developmental changes occur. Support for this supposition comes from the observations that left and right hypoglossal nerves receive a common activation in adult rats but not in the neonatal rat in-vitro preparations, as I reported in Chapter 4. Also, it has been hypothesised that prior to establishing the full complement of descending pathways, motoneurones are anatomically and electrically coupled by gap junctions that act to enhance synchronisation of motor activities through sharing of synaptic inputs (Llinas, 1985; Walton and Navarrette, 1991);
and gap junctions exists between hypoglossal motoneurones in neonatal rats (Mazza et al., 1992). By contrast, I find no evidence that adjacent hypoglossal motoneurones receive or share excitatory inputs arising from the same inspiratory premotor neurones in 1-5 day old rats.

5.4.3 Relationship of Hypoglossal and Phrenic Nerve Activities

In adult rats, I observed that the hypoglossal nerve discharged before the phrenic nerve by ~ 180 ms, but Sica et al. (1984) reported that in cats, hypoglossal nerves only discharge in advance of phrenic nerves by ~ 40 ms. While this difference in reported onset times may reflect species differences, it nonetheless demonstrates that hypoglossal nerves discharge in advance of phrenic nerves, and it has been suggested by some investigators that such different onset times reflect the need for a patent airway preceding inspiration (Iscoe, 1988; Lowe, 1981). By contrast, in the neonatal preparations, I observed that phrenic nerves discharge before hypoglossal nerves by ~ 60 ms on average, in agreement with Morin et al. (1992) and Smith et al. (1990). I view this contrast as further evidence for developmental changes in the motor control of upper airways in rats.

Regardless of how differences between adult and neonatal preparations are interpreted, the observation remains that one nerve often discharges in advance of the other. Two hypotheses might explain this observation. First, hypoglossal and phrenic motoneurones receive inspiratory drive from a common population of premotor neurones, but the transmission times differ. However, in adult rats, the transmission time from the brainstem to phrenic motoneurones is in the order of ~ 2 ms (Tian and Duffin, 1996b), far less than the delay between discharges, and differences in motoneurone excitability appear unlikely to account for a transmission time difference since hypoglossal and phrenic
motoneurones in *in-vitro* brainstem-spinal cord preparations from neonatal rats show no difference in their intrinsic membrane properties (Hilaire and Duron, 1999; Morin et al., 1992). The second hypothesis is that hypoglossal and phrenic motoneurones receive inspiratory drive from different populations of inspiratory premotor neurones, as suggested by Sica et al. (1984) and Morin et al. (1992).

My cross-correlation findings distinguish between these two hypotheses. Cross-correlograms computed between nerves known to receive common excitation exhibit broad central peaks (Duffin and van Alphen, 1995a; Kirkwood and Sears, 1991). However, in both adult and neonatal rat preparations, all cross-correlograms were featureless. I therefore rejected the first hypothesis and concluded that hypoglossal and phrenic motoneurone pools receive inspiratory drives from different inspiratory premotor neurones. My conclusion disagrees with that of Ono et al. (1994, 1998) who suggest that in cats, the premotor drive to phrenic and hypoglossal motoneurones originates from a common source. Because my cross-correlation findings are negative evidence, I therefore sought corroboration. I hypothesised that if phrenic premotor neurones could be identified as monosynaptically exciting phrenic motoneurones by cross-correlating their activity with the discharge of the phrenic nerve, then a monosynaptic connexion to hypoglossal motoneurones should be detectable in the same way. To do this, I examined the functional connexions from identified inspiratory phrenic premotor neurones to hypoglossal motoneurones using cross-correlation analysis.
5.4.4 Characterisation of Inspiratory Phrenic Premotor Neurones

I identified 26 inspiratory phrenic premotor neurones by their location in the medulla, their firing pattern, and the presence of narrow half-amplitude width peaks, at short latencies, in their cross-correlograms computed with the discharge of the phrenic nerve. Twenty-five were located in the ventral respiratory group close to the level of the obex where others have found them (Ezure et al., 1988; Saether et al., 1987; Schwarzacher et al., 1991; Tian and Duffin, 1996b; Zheng et al., 1991), and 1 in the dorsal respiratory group. To date, inspiratory phrenic premotor neurones have not been identified in the dorsal respiratory group of rats (Saether et al., 1987; Tian and Duffin, 1998). All of these neurones had activities confined to the inspiratory phase, augmenting in frequency but declining in amplitude, known attributes of bulbospinal, inspiratory neurones that excite phrenic motoneurones (Ezure et al., 1988; Saether et al., 1987; Schwarzacher et al., 1991; Tian and Duffin, 1996b; Zheng et al., 1991). All showed narrow peaks at short latencies in their cross-correlograms with phrenic activity that I interpreted as evidence for monosynaptic excitatory connexions (Tian and Duffin, 1996b).

5.4.5 Relationship of Inspiratory Phrenic Premotor Neurones and Hypoglossal Motoneurones

Of the cross-correlograms computed between the activity of these 26 inspiratory phrenic premotor neurones and the discharge of the hypoglossal nerve only one displayed a narrow peak at a short latency, evidence that this neurone monosynaptically excited both phrenic and hypoglossal motoneurones; the majority, (25 of 26) did not. I concluded that
phrenic premotor neurones are not the source of inspiratory excitation of hypoglossal motoneurones in rats.

Results from neuroanatomical tracing experiments in adult rats disagree. They show that ventral respiratory group neurones, albeit unidentified in terms of function, project to both phrenic and hypoglossal motor nuclei (Dobbins and Feldman, 1994; Dobbins and Feldman, 1995). Also, Lipski et al. (1994) used a combination of electrophysiological identification and neuroanatomical tracing techniques in adult rats, to demonstrate that 4 of the 9 ventral respiratory group inspiratory neurones examined projected to both the phrenic and hypoglossal motor nuclei. I also found that some inspiratory ventral respiratory group neurones could be antidromically activated from the hypoglossal motor nucleus, but cross-correlation showed no functional connexions with the hypoglossal nerve (see Chapter 6).

The discrepancy between findings from anatomical tracing and antidromic activation studies, on the one hand, and my cross-correlation studies of functional connexions, on the other hand, might be resolved in several ways. First, if the excitation from phrenic premotor neurones is transmitted to hypoglossal motoneurones via a polysynaptic pathway, then cross-correlation might fail to detect the connexion. Second, if the population of neurones I studied was different from those in other studies then different results would also be expected. Third, species differences might explain the different observations.

Evidence in support of the first hypothesis comes from experiments on the isolated respiratory network of the transverse brainstem slice. Wilson et al. (1999) found evidence in neonatal rats (0-5 days old) that inspiratory neurones dorsomedial to the pre-Bötzing complex are premotor to hypoglossal motoneurones. In slices from neonatal mice, it has been observed that the coupling between rhythmic activity in ventral respiratory group
neurones and hypoglossal nerves was approximately 3:1 (Ramirez et al., 1996). This observation could mean that the connexion pathway was not coupled monosynaptically, but it could also mean that the hypoglossal motoneurones do not respond to every excitatory burst. The latter interpretation implies that their membrane properties differ from phrenic motoneurones, which, as discussed previously, they do not.

Evidence for the second hypothesis, that neurones projecting to and exciting both phrenic and hypoglossal motoneurones are not located in the ventral respiratory group where I recorded my phrenic premotor neurones comes from the experiments in cats by Ono et al. (1994). They found both anatomical and functional evidence for common excitation of hypoglossal and phrenic motoneurones. Resolution of these two hypotheses cannot therefore be reached without further experimentation in rats. However, I contend that inspiratory excitation of hypoglossal motoneurones is relayed, at least in part, via interneurones situated within the hypoglossal motonucleus; and this hypothesis will be addressed in the following chapter. Their existence within the hypoglossal motonucleus has been suggested from both electrophysiological and neuroanatomical evidence in rats (Boone and Aldes, 1984; Viana et al., 1994) but their function is unknown. Modulation of the interneurones by other inputs could account for the differences in timing between phrenic and hypoglossal activities. Such modulation is exerted by the pons (St.-John, 1987; St.-John, 1998), and several observations illustrate this point. The rostral pons is responsible for a delay in the onset of the active phases of the phrenic rootlets compared with that of the trigeminal, facial and hypoglossal rootlets in in-vitro brainstem-spinal cord preparations from neonatal mice (Jacquin et al., 1999). During pharmacologically induced rapid-eye movement sleep in decerebrate cats, hypoglossal motoneurones decreased their activity more than phrenic motoneurones (Fenik et
Hypoglossal activity is also decreased during stimulation of midpontine dorsal tegmentum in adult cats (Kawahara et al., 1989). From these observations, it might be suggested that respiratory drive to hypoglossal motoneurones originates from the pons, as suggested by Kuna and Remmers, (1999). Because hypoglossal activity is present in the neonatal in-vitro preparations after the pons is removed, it is therefore unlikely that the pons contains the essential premotor circuitry to transmit respiratory rhythm to hypoglossal motoneurones. Nonetheless, I will test this hypothesis in the following chapter.

Finally, I note the species difference. Neither of the first two explanations is very satisfactory; even with a polysynaptic pathway, cross-correlation should detect connexions as the averaging technique employed by Ono et al. (1994) did, and I explored the medulla thoroughly seeking phrenic premotor neurones and should not have missed a population. That leaves species difference as an explanation, and I note differences between rats and cats already found (for details, see Chapter 1, section 1.10.1); phrenic premotor neurones in rats project bilaterally to monosynaptically excite both left and right phrenic motoneurones, but in cats they project mostly unilaterally to contralateral phrenic motor neurones (Duffin and van Alphen, 1995a); dorsal respiratory group neurones in rats have few spinal projections in contrast to those in cats (Tian and Duffin, 1998).

5.4.6 Relationship of Bötzinger Complex Expiratory Neurones and Phrenic and Hypoglossal Motoneurones

In adult rats, Bötzinger complex expiratory neurones project to and monosynaptically inhibit phrenic motoneurones during expiration (Tian et al., 1998a). The axonal projections of Bötzinger complex expiratory neurones have been well characterised and are known to
have axons clustered in the lateral spinal cord at the second and third cervical level so that stimulation of this region inhibits the discharge of phrenic motoneurones (Tian et al., 1998a). In the present experiment, I confirmed this observation. Post-stimulus histograms of phrenic nerve discharges were of three types, all exhibiting troughs at short latencies indicative of monosynaptic inhibition of phrenic motoneurones. The first type consisted of a trough at a short latency but with otherwise featureless histograms. In the second type, post-stimulus histograms revealed a statistically significant peak preceding the trough, and I interpret these peaks to indicate that excitatory inputs to phrenic motoneurones were also stimulated thereby exciting phrenic motoneurones before Bötzinger complex expiratory neurones inhibited them. In the third type, post-stimulus histograms showed a trough followed immediately by a broad peak, which I suggest, are due to the effects of post-inhibitory rebound excitation.

These descending inputs to phrenic motoneurones would also be antidromically activated by my stimuli. Post-stimulus histograms of hypoglossal nerve discharges computed simultaneously with those for the phrenic should therefore display the same features if these axons had collaterals synapsing with hypoglossal motoneurones. However, the post-stimulus histograms of hypoglossal discharge were featureless. I concluded that neither the inhibitory inputs from Bötzinger complex expiratory neurones, nor the excitatory inputs from descending axons to phrenic motoneurones, monosynaptically project to hypoglossal motoneurones. The latter observation further strengthens my conviction that phrenic inspiratory premotor neurones do not monosynaptically project to and excite hypoglossal motoneurones. That hypoglossal motoneurones are not inhibited by Bötzinger complex expiratory neurones is also supported by findings from other experiments. In cats, inspiratory hypoglossal motoneurones do not appear to be actively inhibited during
expiration (Withington-Wray et al., 1988; Woch and Kubin, 1995); hypoglossal motoneurones may therefore not be actively inhibited during expiration but disfacilitated. This hypothesis will be further tested in the following chapter.

5.4.7 Conclusions

(1) In adult rats, motoneurones of the medial and lateral hypoglossal nerve branches receive inspiratory drive from a common premotor population, but adjacent nerve rootlets in neonatal rats do not. I suggest that the medial and lateral hypoglossal motoneurones receive excitation from a common population of premotor neurones in the adult, but in the neonate the terminal arborisation of these premotor neurones are undeveloped. (2) In both adult and neonatal rats, hypoglossal motoneurones are not monosynaptically excited by the inspiratory neurones that are premotor to phrenic motoneurones. (3) In adult rats, hypoglossal motoneurones are not inhibited by the same neurones, likely Bötzinger complex expiratory neurones, that inhibit phrenic motoneurones. I therefore suggest that respiratory control of hypoglossal motoneurones is separate from that for phrenic motoneurones in rats. In the following chapter, I will characterise the premotor source of respiratory drive transmission to hypoglossal motoneurones.
CHAPTER 6

RESPIRATORY PREMOTOR CONTROL OF HYPOGLOSSAL MOTONEURONES

6.0 SUMMARY

The activity of the hypoglossal nerves displays a respiratory rhythm in rats; intracellular records from 28 hypoglossal motoneurones showed respiratory fluctuations in membrane potential with all neurones depolarising (mean ± standard error, 10 ± 1 mV) during inspiration and repolarising during expiration (resting membrane potential, -50 ± 3 mV). Iontophoresis of chloride ions to reverse inhibitory hyperpolarizations to depolarisations, (25-100 nA min) did not detectably change membrane potential trajectories (n = 21). I sought the origin and transmission pathway for this rhythm. Although removing the pons markedly altered respiratory rhythm in both hypoglossal and phrenic nerves, rhythm was not eliminated, and I concluded that while the pons is important for respiratory rhythm modulation, it is not the origin of hypoglossal nerve respiratory rhythm. I then tested the hypotheses that inspiratory drive is transmitted to hypoglossal motoneurones from premotor neurones in the lateral tegmental field, and ventral respiratory group, possibly via interneurones within the hypoglossal motor nucleus (see figure 6.0), by cross-correlating their activity with that of both the hypoglossal and phrenic nerves. Of the 36 inspiratory neurones recorded within the hypoglossal motor nucleus, 9 (25 %) were identified as motoneurones by antidromic activation of the hypoglossal nerve and from cross-correlogram features, narrow half-amplitude peaks (0.5 ± 0.1 ms) at short latencies (0.2 ± 0.1 ms). The
cross-correlograms of those inspiratory neurones not antidromically activated (75 %) featured either central peaks (1.7 ± 0.2 ms) alone (n = 14; 39 %), or central peaks (1.3 ± 0.2 ms) followed by troughs (1.3 ± 0.1 ms) at short latencies (1.1 ± 0.4 ms) (n = 13; 36 %). I interpret these observations as evidence for interneurones within the hypoglossal motor nucleus. The cross-correlograms for the extracellular recordings from 238 inspiratory within the lateral tegmental field and ventral respiratory group were mostly featureless, but 19 (8 %) displayed narrow half-amplitude peaks (1.0 ± 0.1 ms) at short latencies (0.9 ± 0.1 ms) with the hypoglossal nerve, which is indicative of monosynaptic excitation. I interpret these observations as evidence that these neurones were hypoglossal premotor neurones, located diffusely throughout the lateral tegmental field, dorsal to the ventral respiratory group. I conclude that: (1) hypoglossal inspiratory motoneurones are not postsynaptically inhibited during expiration; (2) respiratory rhythm is not transmitted to hypoglossal motoneurones from the pons; (3) respiratory rhythm is transmitted to hypoglossal motoneurones from a distinct population of premotor neurones scattered throughout the lateral tegmental field; (4) interneurones within the hypoglossal motor nucleus may also participate in the transmission of respiratory rhythm.

6.1 Introduction

Hypoglossal motoneurones innervate the intrinsic and extrinsic tongue muscles (Dobbins and Feldman, 1995; Uemura-Sumi et al., 1988), which serve a wide variety of motor tasks, including breathing (Lowe, 1981; Travers and Jackson, 1992). Understanding the respiratory control of hypoglossal motoneurones is essential because of their critical involvement in maintaining airway patency during normal breathing, and their putative role
in the pathogenesis of obstructive sleep apnoea (Brouillette and Thach, 1980; Fregosi and Fuller, 1997; Horner, 1996; Remmers et al., 1978). Despite such importance in both normal and altered physiological states, virtually nothing is known about how respiratory rhythm is transmitted to hypoglossal motoneurones. To this end, I used a variety of electrophysiological techniques including cross-correlation and lesioning in the decerebrate adult rat to determine the mechanisms and pathways by which respiratory rhythm is transmitted to hypoglossal motoneurones.

Hypoglossal motoneurones are somatotopically organised in bilateral nuclei extending along the midline of the medulla (Dobbins and Feldman, 1995; Uemura-Sumi et al., 1988; Uemura-Sumi et al., 1981). During the inspiratory phase of the respiratory cycle, hypoglossal motoneurones are synaptically excited, so that tongue muscles are activated and stiffened to maintain a patent pharyngeal airway, allowing the diaphragm to draw air into the lungs (Brouillette and Thach, 1980; Fregosi and Fuller, 1997; Remmers et al., 1978). Thus, the rhythmic contractions of the upper airway and diaphragm, which are controlled by a network of neurones located within the medulla and pons (Duffin et al., 1995; St.-John, 1998) must occur in an appropriate sequence (Iscoe, 1988; Sica et al., 1984). Although the premotor circuitry for phrenic motoneurones innervating the diaphragm are well understood (Monteau and Hilaire, 1991; Parkis et al., 1999; Tian et al., 1998a), knowledge of the respiratory premotor control of hypoglossal motoneurones is surprisingly deficient.

In cats and rats, bulbospinal inspiratory neurones located within the ventral respiratory group transmit inspiratory drive to phrenic motoneurones (Lui et al., 1990; Monteau and Hilaire, 1991; Parkis et al., 1999; Tian and Duffin, 1996b) and bulbospinal Bötziinger complex expiratory neurones actively inhibit phrenic motoneurones during
expiration (Merrill and Fedorko, 1984; Tian et al., 1998a). While most respiratory neurones are actively inhibited during expiration, not all respiratory-modulated motoneurones are inhibited (Parkis et al., 1999; Woch and Kubin, 1995). In the previous chapter, I demonstrated that in adult rats, hypoglossal motoneurones are not actively inhibited by bulbospinal Bötzinger complex expiratory neurones (Peever et al., 2000). This observation is in agreement with reports that hypoglossal motoneurones in cats are not postsynaptically inhibited during expiration (Withington-Wray et al., 1988; Woch and Kubin, 1995). Such differences suggest that the underlying organisations of premotor pathways transmitting respiratory drive to hypoglossal and phrenic respiratory motoneurones differ, as suggested in Chapter 5. I therefore made intracellular recordings from inspiratory-modulated hypoglossal motoneurones to assess the mechanisms by which hypoglossal motoneurone membrane trajectories are synaptically altered during the respiratory cycle; using chloride iontophoresis to reverse hyperpolarization to depolarisation and determine whether hypoglossal motoneurones are postsynaptically inhibited during the respiratory cycle.

Detailed neuroanatomical studies describe a morphologically distinct population of non-glial cells located within the hypoglossal motor nucleus; they are distinguishable from hypoglossal motoneurones, and are presumed to be interneurones (Boone and Aldes, 1984; Cooper, 1981; Takasu and Hashimoto, 1988; Takasu et al., 1987; Takata, 1993). Electrophysiological studies in cats report long-latency synaptic potentials recorded from neurones located within the hypoglossal motor nucleus, and Sumi (1969) and Takata (1993) suggests that such a response is indicative of hypoglossal interneurones. Despite the identification of putative hypoglossal interneurones, to date no studies have determined their connectivity or function. I hypothesised that hypoglossal interneurones make functional
connexions with hypoglossal motoneurones (see figure 6.0), and like thoracic interneurones, transmit respiratory drive to hypoglossal motoneurones (Kirkwood et al., 1993; Sears, 1964b). To test this hypothesis, I made extracellular recordings of non-antidromically activated inspiratory-modulated hypoglossal neurones (see figure 6.1) and computed cross-correlograms between their activity and that of the hypoglossal nerve to detect functional connexions.

In rats and cats, neuroanatomical evidence demonstrates that the medulla, pons, and cortex contain neurones projecting to hypoglossal motoneurones (Borke et al., 1983; Dobbins and Feldman, 1995; Holstege et al., 1977; Li et al., 1993; Sahara et al., 1996; Takada et al., 1984; Ugolini, 1995). Specifically, these studies indicate that hypoglossal motoneurones receive projections from the lateral tegmental field, raphé nuclei and Kölliker-Fuse nucleus of the rostral pons. In rats, projections to hypoglossal motoneurones were detected by exposing the medial and lateral hypoglossal nerve branches to pseudo rabies virus; in this study, Dobbins and Feldman, (1995) showed that hypoglossal motoneurones receive projections predominantly from the lateral tegmental field as well as the pontine Kölliker-Fuse nucleus. Recently, Kuna and Remmers (1999) confirmed the latter projection in cats. Using spike-triggered averaging of hypoglossal discharge, it has also been suggested that in cats inspiratory neurones ventrolateral to the hypoglossal motor nucleus and in close proximity to the ventral respiratory group synaptically excite hypoglossal motoneurones (Ono et al., 1994). Based on these reports, I hypothesised that respiratory rhythm was transmitted to hypoglossal motoneurones from neurones within the lateral tegmental field, ventral respiratory group and pons (see figure 6.1). Removing the pons and observing the effect on hypoglossal nerve activity tested the latter possibility, while the first two
Figure 6.0. A schematic representation of the putative premotor inputs to hypoglossal (XII) motoneurones in the adult rat. It hypothesise that respiratory rhythm is transmitted to XII motoneurones by: A, interneurones situated within the XII motor nucleus; B, premotor neurones in the pons; C, premotor neurones in the nucleus ambiguus (NA); and D, premotor neurones in the lateral tegmental field (LTF).
possibilities were tested using cross-correlation of the extracellular activity of neurones recorded within the lateral tegmental field and ventral respiratory group with that of the hypoglossal and phrenic nerves to detect functional connexions.

My observations led me to conclude that in adult rats hypoglossal motoneurones are not actively inhibited during expiration, and that they receive inspiratory drive from a distinct population of inspiratory premotor neurones within the lateral tegmental field and perhaps from a population of interneurones within the hypoglossal motor nucleus.

6.2 METHODS

6.2.1 Animal Preparation

Adult decerebrate rats were used in these experiments and were prepared as described in Chapter 2, section 2.1.1.

6.2.2 Nerve and Cellular Recordings

Nerves and individual neurones were recorded as described in Chapter 2, section 2.3.1. Example recordings are illustrated in figures 6.2.

6.2.3 Protocols

Figure 6.1 depicts the general protocols used in these experiments.

6.2.3.1 Hypoglossal Inspiratory Motoneurones

After establishing stable recordings of hypoglossal and phrenic nerves, I stimulated the hypoglossal nerve (pulses: 0.2 ms wide, 2-4 V, 5 Hz) and used the resulting field potential to locate the hypoglossal motor nucleus (see figure 6.1A). Then, intra- or
Figure 6.1. A schematic representation of the nerves, and neurones that I recorded in the medulla and spinal cord of the adult rat; the putative connexions of recorded neurones are illustrated. Whole-nerve recording were made from ipsilateral hypoglossal (XII) and phrenic (C4) nerves. Extracellular recording were made from inspiratory neurones in the: XII motor nucleus, XII-I; nucleus ambiguus/ventral respiratory group, VRG-I; lateral tegmental field, LTF-I. Intracellular recording were made from XII motoneurones (Mn). The pons was removed (shaded area) by suctioning all tissue 4-5 mm rostral of the obex.
extracellular searches were made for inspiratory neurones within the identified region. I identified hypoglossal motoneurones by antidromically activating them from the ipsilateral hypoglossal nerve (pulses: 0.2 ms, 2-10 V, 5 Hz). Collision tests were performed to verify the antidromic nature of the activation (Lipski, 1981). In addition, I computed cross-correlograms between the inspiratory neurones' discharge and that of the ipsilateral hypoglossal and phrenic nerves to detect functional connexions (Kirkwood, 1979). For those inspiratory neurones identified as hypoglossal motoneurones, I monitored their intracellularly recorded membrane potential trajectories for several minutes, and then iontophoretically injected chloride ions (5-10 nA) for 5-10 minutes to reverse inhibitory hyperpolarizations to depolarisations and detect postsynaptic inhibitory inputs.

6.2.3.2 Removal of the Pons

After obtaining stable baseline recordings of hypoglossal and phrenic nerves for a minimum of 5 min, all structures 4-5 mm rostral to obex were rapidly aspirated using suction (figure 6.1). The procedure was carried out under observation with a dissection microscope (Ziess, OPMI 1-FC), and to help stop bleeding very small balls (~5 mm diameter) of cotton soaked with thrombin were gently packed into the base of the skull. Approximately 5-15 min passed before hypoglossal and phrenic nerve activities were recorded again.

6.2.3.3 Inspiratory-Modulated Lateral Tegmental Field and Ventral Respiratory Group Neurones

In initial experiments, stimulating microelectrodes were stereotaxically positioned throughout the mediolateral and rostrocaudal co-ordinates (Paxinos and Watson, 1986) of the
hypoglossal motor nucleus. I hypothesised that delivered stimuli (pulses: 0.2 ms, 10-30 μA) would antidromically activate axons projecting to the hypoglossal motor nucleus, and produce field potentials in the lateral tegmental field and ventral respiratory group so that inspiratory neurones could be located. Collision tests were performed to verify the antidromic nature of the activation (Lipski, 1981). Inspiratory neurones identified as projecting to the hypoglossal motor nucleus were then tested for functional connexions to hypoglossal and phrenic motoneurones by cross-correlating their discharges with those of the phrenic and hypoglossal nerves (Kirkwood, 1979).

In later experiments, I recorded extracellularly from inspiratory neurones throughout the medulla (see figure 6.1 and section 6.2.4 for the method used to classify them). Neurons located in close proximity to the hypoglossal motor nucleus were tested for antidromic activation by stimulating (pulses: 0.2 ms, 2-10 V, 5 Hz) the ipsilateral hypoglossal nerve to identify them as hypoglossal motoneurones; and most neurones (~ 60%) in close proximity to the ventral respiratory group (see section 6.2.4, Stereotaxic Classification of Neurones) were tested for antidromic activation by stimulating the ipsilateral vagus nerve to identify them as vagal motoneurones. Those inspiratory neurones not antidromically activated from either the hypoglossal or vagus nerves were cross-correlated with the inspiratory activity of hypoglossal and phrenic nerves to detect functional connexions (Kirkwood, 1979).

6.2.4 Data Analysis

Classification of Respiratory Neurones: Neurones were classified based on their location within the medulla and whether they could be antidromically activated from the hypoglossal nerve. Hypoglossal motoneurones were identified by their location within the
hypoglossal motor nucleus as defined by Paxinos and Watson (1986), and by their antidromic activation from the ipsilateral hypoglossal nerve. In this study, the stereotaxic co-ordinates of the hypoglossal motor nuclei are illustrated by the blue boxes in figure 6.3. These blue boxes encompass the location of all antidromically activated hypoglossal motoneurones. Inspiratory neurones that could not be antidromically activated by the ipsilateral hypoglossal nerve that were located within (or at the border of) the hypoglossal motor nuclei were considered hypoglossal interneurones.

The stereotaxic co-ordinates of the ventral respiratory group (see figure 6.3, red boxes) were previously determined by mapping the locations of bulbospinal inspiratory neurones relative to the obex in male adult rats of comparable sizes to those used in these experiments (Tian et al., 1998c). The stereotaxic location of the ventral respiratory group is illustrated in figure 6.3; all neurones situated within this region were classified as ventral respiratory group inspiratory neurones.

According to Paxinos and Watson (1986), nucleus raphé obscurus is located ventral to the hypoglossal motor nuclei. Therefore, neurones situated close to the midline (obex) and ventral to the stereotaxically defined hypoglossal motor nucleus were considered inspiratory raphé neurones (see figure 6.3, green boxes).

All neurones situated lateral and ventrolateral to the hypoglossal motor nuclei, and dorsolateral and dorsomedial to the ventral respiratory group were considered neurones, which belonged to the lateral tegmental field (see figure 6.3).

Intracellular Recordings and Chloride Reversal: Membrane potential trajectories (mV) were correlated with the three stages of the respiratory cycle (inspiration, and early and late expiration) before and after chloride iontophoresis.
Features were also described by their latency to the start, and the half-amplitude width of the feature; values are expressed as mean ± standard error. Unpaired student $t$-tests were used to compare the latencies of cross-correlogram peaks between antidromically activated hypoglossal motoneurones and lateral tegmental field inspiratory neurones. The level of confidence used was $P < 0.05$. Paired $t$-tests were used to compare respiratory timing variables before and after the pons was removed, and unpaired student $t$-tests were used to compare hypoglossal and phrenic nerve timing variables. A confidence level of $P < 0.05$ was used.

6.3 RESULTS

6.3.1 Inspiratory Hypoglossal Neurones

6.3.1.1 Locations and Patterns

I used the antidromically-evoked field potentials (pulses: 0.2 ms wide, 0.2 V, 5 Hz) (figure 6.2A) produced by stimulating the hypoglossal nerve as a guide in my search for inspiratory neurones within the hypoglossal motor nucleus. Neurons with 4 different discharge patterns were encountered. While exact counts were not made, I nonetheless report that the majority were inspiratory, some were expiratory and very few were early expiratory, or tonic. Due to the specific hypothesis being tested, I confined my examination to inspiratory neurones ($n = 64$).

6.3.1.2 Intracellular Recordings

Twenty-eight inspiratory neurones that were antidromically activated (figure 6.2B) from the ipsilateral hypoglossal nerve had resting membrane potentials that ranged from −80
Figure 6.2. Hypoglossal (XII) motoneurone intracellular recordings. A) An example of a field potential used to locate XII motoneurones. It was recorded with an intracellular microelectrode and evoked by XII nerve stimulation with 2 V pulses of 0.2 ms duration at a rate of 5 Hz. The filled circle indicates the stimulus artefact. B) An example of an antidromically activated XII motoneurone. It was recorded with an intracellular microelectrode, and the stimulus was as described in A. The filled circle indicates the stimulus artefact. C and D) Two examples of intracellular recordings from XII motoneurones and corresponding extracellular recordings of the XII nerve. In each, the top two traces show the membrane potential trajectories (upper) and the inspiratory activity of the XII nerve (lower) and the bottom two traces show the same two traces after chloride (Cl⁻) iontophoresis (5 µA for 6 min and 10 µA for 10 min, respectively).
to -20 mV (-52 ± 3 mV). All showed membrane potential fluctuations (range: 5 to 20 mV) with a respiratory pattern related to that observed in the discharge of both hypoglossal and phrenic nerves (figure 6.2C, D). All depolarised during inspiration (10 ± 1 mV) and repolarised during expiration (figure 6.2C, D). In 10 neurones, 5 nA of chloride current was injected for 5-6 min but had no effect on their membrane trajectories (figure 6.2C), nor did 10 nA of chloride injected for 6-10 min in 8 neurones (figure 6.2D). I mapped their positions relative to obex (figure 6.3) using the co-ordinates read from the micromanipulator.

6.3.1.3 Extracellular Recordings

I recorded extracellularly from 36 inspiratory neurones and mapped their positions relative to obex (figure 6.3); 9 (25 %) were antidromically activated from the ipsilateral hypoglossal nerve and collision tests performed (Lipski, 1981) (figure 6.4). Cross-correlograms (mean bin count, 1687 ± 620) computed between their discharge and that of the ipsilateral hypoglossal nerve revealed peaks with narrow half-amplitude widths (0.5 ± 0.1 ms) at short latencies (0.2 ± 0.1 ms) (figure 6.4).

The remaining 27 inspiratory neurones were not antidromically activated from the ipsilateral hypoglossal nerve despite using stimuli of up to 10 V. Cross-correlograms computed between these neurones and the ipsilateral hypoglossal nerve were of 2 types (figure 6.5) and were clearly unlike those for antidromically activated hypoglossal motoneurones (figure 6.4C). The first type of cross-correlogram (n = 14, 39 %; mean bin count, 2129 ± 289) displayed central peaks (mean half-amplitude widths, 1.7 ± 0.2 ms) (figure 6.5C, D), while the second type (n = 13, 36 %; mean bin count, 3375 ± 1473) displayed central peaks (mean half-amplitude widths, 1.3 ± 0.2 ms) followed by troughs (1.3 ± 0.1 ms) at short latencies (1.1 ± 0.4 ms) (figure 6.5A, B). The half-amplitude widths of
Figure 6.3. The micromanipulator co-ordinate locations of the 274 extracellularly recorded inspiratory neurones relative to the surface of the medulla and the obex. A) Dorsal view. B) Transverse view showing the location of the stereotaxically defined ventral respiratory group (red boxes), hypoglossal (XII) motor nuclei (blue boxes), and midline raphé nucleus (green box). The legend indicates the functional relationship of each neurone. They were determined from interpretations of cross-correlograms computed between the activity of each neurone and those of the hypoglossal (XII) and phrenic (C4) nerves (see text for details). Abbreviations used are: Mn, motoneurone; AA, antidromically activated from the ipsilateral XII nerve; LTF, lateral tegmental field; HFO, high-frequency oscillations.
Figure 6.4. Hypoglossal (XII) motoneurone extracellular recordings. A) An example of the extracellular activity of an inspiratory XII motoneurone (top) and the inspiratory discharge of the XII nerve (bottom). B) An example of a collision test demonstrating the antidromic activation of a XII motoneurone (filled circles) from the ipsilateral XII nerve; a, uncollided; b, collided; arrows indicate stimulus artefacts. C) Examples of cross-correlograms computed between an antidromically identified XII motoneurone and the ipsilateral XII nerve. Bin width is 0.2 ms. I interpreted these narrow peaks at short latencies as evidence for the appearance of the action potential recorded at the motoneurone in the nerve discharge.
Figure 6.5. Representative cross-correlograms computed between non-antidromically-activated inspiratory hypoglossal (XII) neurons and the ipsilateral XII nerve. Bin widths are 0.2 ms. A and B) Cross-correlograms displaying broad central peaks followed by narrow troughs at short latencies. I interpreted these features as evidence for a common excitation of the neurone and XII motoneurones, and an inhibition of XII motoneurones by the neurone respectively. C and D) Cross-correlograms displaying broad central peaks only. I interpreted these features as evidence for common activation of the neurone and the XII motoneurones.
both the first and second type of cross-correlogram were significantly wider \((P = 0.001\) and \(P = 0.005\), respectively) than for antidromically activated hypoglossal motoneurones.

### 6.3.2 Effects of Removing the Pons on Hypoglossal and Phrenic Nerve Activities

In 4 of 12 rats, removal of the pons resulted in the discharge of both hypoglossal and phrenic nerves becoming irregular and of sufficiently poor quality that accurate measurement of respiratory timing variables could not be obtained. In 8 of 12 rats, respiratory motor output of the hypoglossal and phrenic nerves was markedly altered but never abolished following removal of the pons (figure 6.6).

Prior to its removal, both hypoglossal and phrenic nerves exhibited inspiratory discharge at a rate of \(60 \pm 7 \text{ b/min}\) (figure 6.6), with the hypoglossal nerve discharging \(0.16 \pm 0.03\) s before the phrenic nerve. The time for one complete respiratory cycle to occur was \(1.68 \pm 0.18\) s and was identical for both hypoglossal (figure 6.7A) and phrenic nerves. The expiratory period and inspiratory duration for the phrenic nerve were \(1.25 \pm 0.14\) s and \(0.44 \pm 0.05\) s, respectively. Expiratory period for the hypoglossal nerve was \(1.10 \pm 0.14\) s (figure 6.7A) and is significantly shorter \((P = 0.008)\) than that for the phrenic nerve, whereas the inspiratory period \((0.58 \pm 0.05\) s) (figure 6.7A) was significantly longer \((P = 0.007)\) for the hypoglossal nerve than for the phrenic nerve. The rise time for the phrenic nerve was \(0.43 \pm 0.04\) s and for the hypoglossal nerve was \(0.42 \pm 0.07\) s (figure 6.7C); not significantly different from one another \((P = 0.873)\).

Following removal of the pons (figure 6.6), the onset times of the hypoglossal and phrenic nerves were identical (figure 6.6), as were the inspiratory and expiratory periods of both nerves the same. After the pons was removed, the cycle length of both hypoglossal and
Figure 6.6. The effect of pons removal on hypoglossal (XII) and phrenic (C4) nerve activities. A, B, and C) Three examples of raw (top traces) and integrated (bottom traces) neurograms of XII and C4 nerve activities before (left) and after (right) removing the pons. Note the profound decrease in inspiratory bursting frequency, total cycle length, and expiratory and inspiratory periods following removal. ‘Non-respiratory’ bursts are visible during the expiratory periods in both B and C.
Figure 6.7. The effect of pons removal on respiratory cycle timing. A) A comparison of respiratory timing variables for the hypoglossal (XII) nerve; total cycle length ($t_{TOT}$), expiratory period ($t_e$), and inspiratory period ($t_i$) both before (open bars) and after (filled bars) removal of the pons. B) An example illustrating the calculation of burst rise time for XII and phrenic (C4) inspiratory bursts. In 2 integrated inspiratory XII bursts both before and after the pons was removed, the point at which integrated discharge reached 80% of peak amplitude was divided by the $t_i$ to determine the rise time. C) A comparison of the rise times of XII nerve inspiratory discharge both before (open bars) and after (filled bars) removal of the pons. Values are expressed as means (+ SE). Stars indicate statistically significant differences between conditions; $P < 0.05$. 
phrenic nerve was significantly increased to 13.0 ± 3.9 s (P = 0.008, and was measured for hypoglossal nerves before and after pons removal), as were both the expiratory and inspiratory periods, which increased to 9.81 ± 3.27 s (P = 0.001) and 3.16 ± 0.79 s (P = 0.001), respectively (figure 6.7A). Respiratory frequency decreased significantly to 17 ± 6 b/min (P = 0.001) (figure 6.6A, B, C), and the rise time of both nerves significantly decreased to 0.11 ± 0.04 s (phrenic; P = 0.001) and 0.15 ± 0.06 s (hypoglossal; P = 0.001).

In 3 of the 8 rats, removal of the pons resulted in bursts of variable duration and amplitude, which appeared irregularly during the expiratory period at an average frequency of 13 ± 4 b/min (figure 6.6C). In 1 rat, short duration low amplitude bursts at regular intervals appeared during the expiratory period of the phrenic nerve, and also appeared occasionally in the hypoglossal nerve (figure 6.6B).

6.3.3 Inspiratory Neurones of the Lateral Tegmental Field and Ventral Respiratory Group

In initial experiments (n = 5), I attempted to use antidromic field potentials (e.g., figure 6.2A) evoked from a stimulating electrode positioned at various co-ordinates within the hypoglossal motor nucleus to search for inspiratory neurones with projections to the hypoglossal motor nucleus. I found little evidence of antidromically induced field potentials within the lateral tegmental field, or ventral respiratory group, and abandoned such a searching strategy. Only 6 of 238 (2.5 %) inspiratory neurones within the lateral tegmental field were antidromically activated from within the hypoglossal motor nucleus; their cross-correlograms computed between their discharge and that of the ipsilateral hypoglossal nerve were featureless (figure 6.9).
Two-hundred and thirty-eight inspiratory neurones were located relative to the obex, within the lateral tegmental field, ventral respiratory group and very few within the midline raphé nuclei (figure 6.3). None were antidromically activated from either the ipsilateral hypoglossal or vagal nerves. Cross-correlograms were computed between the discharge recorded from these neurones and that of the phrenic and hypoglossal nerves, and the histograms of 29 of the 238 (12%) neurones displayed features. Nineteen (8%, 15 ipsilateral, 4 contralateral) were computed between inspiratory neurones of the lateral tegmental field (n = 18) and ventral respiratory group (n = 1) with the hypoglossal nerve (figure 6.3). They showed narrow peaks (mean half-amplitude widths, 1.0 ± 0.1 ms; mean bin count, 2495 ± 604) at short latencies (0.9 ± 0.1 ms) (figure 6.8) that were significantly wider (P = 0.027) and longer (P = 0.001) than those measured for inspiratory neurones antidromically activated from the hypoglossal nerve. None of the cross-correlograms computed between the discharge of these neurones and phrenic nerve activity displayed significant features (mean bin count, 2597 ± 658).

Cross-correlograms computed between the activity of 10 inspiratory neurones located variously within the lateral tegmental field (n = 3), ventral respiratory group (n = 4) and midline raphé nuclei (n = 3) and that of the phrenic nerve, revealed periodic peaks and troughs, indicative of high-frequency oscillations, with a mean inter-peak interval of 8.4 ± 0.7 ms (figure 6.10) and high-frequency oscillations frequency of 121 ± 6 Hz. Only 1 neurone located in the lateral tegmental field revealed high-frequency oscillations with both phrenic and hypoglossal nerves.
Figure 6.8. Representative cross-correlograms computed between the discharge of inspiratory neurones in the lateral tegmental field (LTF-I) and that of the hypoglossal (XII) nerve. I interpreted the narrow peaks at short latencies as evidence for a monosynaptic excitation of XII motoneurones. Bin widths are 0.2 ms.
Figure 6.9. An example of an inspiratory neurone in the lateral tegmental field (LTF) antidromically activated from the hypoglossal (XII) motor nucleus but whose activity was not correlated with that of the XII nerve (1 of 5). A) The extracellular activity of the inspiratory neuron located in the LTF (LTF-I) (top) and the inspiratory discharge of the XII nerve (bottom). B) Collision tests demonstrating the antidromic activation of the LTF-I neurone (filled circles) from a stimulating electrode placed within the ipsilateral XII motor nucleus; a, uncollided; b, collided; arrows indicate stimulus artefacts. C, The cross-correlogram computed between the activity of the antidromically identified LTF-I neurone and that of the ipsilateral XII nerve. I interpreted the lack of features in the cross-correlogram as evidence for a lack of a functional connection from the neurone to the XII motoneurones; while this inspiratory neurone projects to the XII motor nucleus it does not directly excite XII motoneurones. Bin widths are 0.2 ms.
Figure 6.10. Examples of high-frequency oscillations (HFO). A) Cross-correlograms computed between the discharge of an inspiratory neurone located in the lateral tegmental field (LTF-I) with the activities of the hypoglossal (XII; left) and phrenic (C4; right) nerves. B) Cross-correlograms computed between the discharge of an inspiratory neurone located in the ventral respiratory (VRG-I) with the activities of the XII (left) and C4 (right) nerves. I interpreted the peaks and troughs at regular intervals as HFO. This example was the only 1 of 9 cases where HFO was observed in cross-correlograms with the XII nerve.
6.4 DISCUSSION

Intracellular recordings from hypoglossal motoneurones showed the membrane potential depolarising during inspiration, with repolarisation during expiration due to disfacilitation rather than inhibition. I conclude that hypoglossal motoneurones are synaptically excited during inspiration but are not actively inhibited during expiration. Although removal of the pons markedly affected respiratory cycle timing of the discharge in both hypoglossal and phrenic nerves, rhythm persisted. I conclude that respiratory drive to hypoglossal motoneurones does not originate in the pons. Cross-correlograms computed between inspiratory neurones located both within the hypoglossal motor nucleus and the lateral tegmental field displayed features I interpreted as evidence for functional connexions. I concluded that respiratory drive to hypoglossal motoneurones originates from 2 sources; inspiratory neurones of the lateral tegmental field, and inspiratory interneurones within the hypoglossal motor nucleus. A full discussion of these findings, their interpretation and their functional significance follows.

6.4.1 Inspiratory Hypoglossal Neurones

The respiratory cycle consists of three distinct phases of neural activity: inspiration, and stages 1 (early) and 2 (late) of expiration; these phases are associated with particular patterns of postsynaptic excitation and inhibition of phrenic motoneurones (Duffin et al., 2000). Similarly, within the hypoglossal motor nucleus I report the presence of neurones with 4 distinct patterns of neuronal discharge correlated with particular phases of the respiratory cycle: the majority inspiratory, some expiratory, and very few early expiratory, and tonic. In cats, Hwang et al. (1983a) report hypoglossal neurones with similar discharge
patterns, but found that most were only active under hypercapnic (expired end-tidal CO\textsubscript{2}, 6-9 \%) or hypoxic (expired end-tidal O\textsubscript{2}, 8-12 \%) conditions. While I did not aim to measure the effects of varying levels of CO\textsubscript{2} on hypoglossal neuronal discharge I found that the hypoglossal nerve rarely discharged at end-tidal CO\textsubscript{2} levels below 4 \%, and therefore maintained end-tidal CO\textsubscript{2} levels between 4-7 \%.

I confined my attention to inspiratory neurones and divided them into two types; those antidromically activated from the ipsilateral hypoglossal nerve and those not activated. The former I took to be hypoglossal motoneurones and the latter hypoglossal interneurones. While antidromic activation constitutes a robust identification of motoneurones the lack does not necessarily identify interneurones. I therefore used cross-correlograms computed between the neurone and hypoglossal nerve discharge as a further aid to identification.

Cross-correlograms for neurones identified by antidromic activation differed markedly from those not activated; the former displayed narrow peaks at short latencies confirming the presence of motoneurones action potentials in the hypoglossal nerve discharge. The cross-correlograms for non-activated neurones did not display such peaks but were characterised by broad central peaks, sometimes followed by a trough, which I interpreted as indicating a common activation of the neurone and hypoglossal motoneurones and an inhibition of hypoglossal motoneurones by the inspiratory neurone respectively. The antidromically-activated neurones lacked a broad central peak indicating that their inspiratory excitation did not originate from a common source. My interpretations of the cross-correlogram features are consistent with those made previously (Duffin and Iscoe, 1996; Duffin and van Alphen, 1995b; Feldman and Speck, 1983; Kirkwood, 1979; Kirkwood and Sears, 1991; Li et al., 1999). To further reinforce my final interpretation, I used a recently
developed cross-correlation simulation program (Duffin, 2000) to mimic the effects two neurones receiving a common excitation while one neurone inhibits the other; such a simulation produced cross-correlograms (see figure 6.11) that were strikingly similar to those presented here (see figure 6.5 and 6.11).

While these observations do not unambiguously identify the non-activated neurones as interneurones, I suggest that they are, and may be involved in the respiratory control of hypoglossal motoneurones. The cross-correlation histogram features are consistent with this view; common activation was observed for the putative interneurones not the motoneurones and some putative interneurones appear to inhibit hypoglossal motoneurones, a function unlikely to occur between two motoneurones.

The membrane potentials recorded from inspiratory hypoglossal motoneurones all displayed respiratory modulation, depolarising during inspiration and rapidly repolarising at the start of expiration. I could not detect any changes in the membrane potential trajectories of these neurones when I iontophoretically injected chloride ions into these motoneurones, and so while I cannot rule out the presence of a reversed inhibitory hyperpolarization during inspiration I am confident that such did not occur during expiration. In cats, hypoglossal motoneurones exhibit similar membrane trajectories, which result from a combination of postsynaptic excitation and inhibition during inspiration, with little or no inhibition during expiration (Withington-Wray et al., 1988; Woch and Kubin, 1995). This finding is supported by my recent observation that Bötzinger complex expiratory neurones, known to inhibit many types of respiratory neurones, including themselves and phrenic motoneurones, (Tian et al., 1998a; Tian et al., 1998b; Tian et al., 1999b) do not inhibit hypoglossal motoneurones
Figure 6.11. A, a cross-correlogram computed from a recently developed simulation program (Duffin, 2000). It was produced by simulating two neurones receiving a common excitation while one neurone inhibited the other. B, is the same cross-correlogram as reported in figure 6.5 A, which was computed from the discharge activity of a non-antidromically activated inspiratory hypoglossal (XII) neurone and the XII nerve.
(see Chapter 5). Inspiratory facial motoneurones in the rat (Huangfu et al., 1993) do not receive inhibition during expiration either.

I concluded that inspiratory-modulated hypoglossal motoneurones are postsynaptically excited during inspiration and are disfacilitated rather than postsynaptically inhibited during expiration in rats. This apparent lack of expiratory inhibition distinguishes hypoglossal motoneurones from phrenic and thoracic respiratory motoneurones, which both receive postsynaptic inhibition during expiration (Milano et al., 1992; Sears, 1964a; Tian et al., 1998a), and suggests that the principles underlying the organisation of premotor pathways transmitting respiratory inputs to hypoglossal and phrenic respiratory motoneurones differ. This contention further supports my observations in Chapter 5, that different premotor populations control phrenic and hypoglossal motoneurones. I suggest that the lack of expiratory inhibition may enhance the control of hypoglossal motoneurones during expiration when they are activated by non-respiratory inputs; all forms of phonation are achieved entirely during the expiratory phase (see Chapter 7, General Discussion).

6.4.2 Pons and Respiratory Rhythm

Neuroanatomical tracing studies in both rat and cat identify many possible premotor sources for hypoglossal motoneurones (Borke et al., 1983; Dobbins and Feldman, 1995; Holstege et al., 1977; Li et al., 1993; Sahara et al., 1996; Takada et al., 1984; Ugolini, 1995), including the pontine Kölliker-Fuse nuclei, and the lateral tegmental field and ventral respiratory group. These regions contain neurones with respiratory-modulated discharge patterns (De Castro et al., 1994; Dick et al., 1994; Saether et al., 1987; Vibert et al., 1976), and when stimulated either electrically or chemically, alter either phrenic or hypoglossal
motor output (Dutschmann and Herbert, 1996; Lowe, 1978; Ramirez et al., 1998; St.-John, 1986a; St.-John, 1986b). It is unlikely that the pons contains the essential premotor circuitry to transmit respiratory rhythm to hypoglossal motoneurones because in adult and neonatal rat preparations without an intact pons, hypoglossal motoneurones continue to exhibit respiratory motor output (Hilaire et al., 1989; Smith et al., 1990). Nevertheless, Kuna and Remmers (1999) suggest that hypoglossal motoneurones receive a selective premotor input from the pontine Kolliker-Fuse nucleus.

My technique for removing the pons employed suction under visual inspection via an operating microscope, and was not without unintended consequences such as excessive blood loss in some cases. I stress however that such trauma was detectable and hence 4 of these preparations were eliminated from analysis. In the other 8 rats, there was minimal bleeding and oedema, and so I am confident that the changes in respiratory activity observed were the result of removal of the pons rather than medullary trauma.

I found that complete removal of all structures 4-5 mm rostral to obex, including the pons had a marked affect on the expression of respiratory motor output of both hypoglossal and phrenic nerves. The characteristic features of removing the pons were dramatic reductions in the frequency of neural inspirations, due primarily to an increase in the length of the expiratory period, and significant increases in the duration of inspiration; an observation similar to those previously reported in rats (Jodkowski et al., 1994; Wang et al., 1993), but in contrast to that reported by others (Monteau et al., 1989). Prior to removing the pons, the inspiratory duration was significantly shorter and expiration duration longer for the hypoglossal nerve than they were for the phrenic nerve. The difference between hypoglossal and phrenic nerve timing variables was due to the advanced onset of hypoglossal nerve
discharge relative to the phrenic nerve; the hypoglossal nerve discharging 0.16 ± 0.03 s in advance of the phrenic nerve, a result consistent with the observations presented in the previous chapter (Peever and Duffin, 2000). However, after the pons was removed, not only did hypoglossal and phrenic nerve discharged in phase with one another, but the periods of inspiratory and expiratory phases were the same for both nerves. In in-vitro brainstem-spinal cord preparations from neonatal rats and mice, removal of the pons either slows (McLean and Remmers, 1994) or has no affect on respiratory rhythm (Smith et al., 1991). It also alters the relative onset times of hypoglossal and phrenic nerves (Jacquin et al., 1999; Smith et al., 1990) such that the hypoglossal nerve discharges in advance of the phrenic after the pons is removed.

From these observations, I conclude that the pons is involved in the expression of respiratory motor output, and while it may contain respiratory-related neurones that project to hypoglossal motoneurones (Kuna and Remmers, 1999), these neurones do not provide the essential respiratory drive to hypoglossal motoneurones.

Not only was respiratory rhythm markedly affected after the pons was removed, but the emergence of a ‘non-respiratory’ pattern of neural activity became apparent. In 3 cases, short duration variable amplitude bursts appeared irregularly during the expiratory period of the hypoglossal nerve; in 1 case, regular low-amplitude bursts appeared during the expiratory period of phrenic nerve discharge. I speculate that the removal of inhibitory inputs from the pons (Hilaire et al., 1989) may have lowered the threshold for motoneurone excitability, thereby allowing the expression of other brainstem pattern-generators such as the swallow-generating network (Lowe, 1981).
6.4.3 Inspiratory Neurones of the Lateral Tegmental Field and Ventral Respiratory Group

I found 19 inspiratory neurones whose cross-correlograms computed between their discharge and that of the hypoglossal nerve exhibited narrow mean half-amplitude peaks at short latencies, which I interpret as indicating monosynaptic connexions. While numerous neuroanatomical studies have shown that neurones located in the lateral tegmental field project to the hypoglossal motor nucleus (Borke et al., 1983; Dobbins and Feldman, 1995; Holstege et al., 1977; Li et al., 1993; Sahara et al., 1996; Takada et al., 1984; Ugolini, 1995), they were not identified nor were their functional connexions verified to distinguish them as hypoglossal premotor neurones as I have done here.

The hypoglossal premotor neurones were distributed bilaterally (15 ipsilateral, 4 contralateral), although the apparent ipsilateral dominance likely resulted because 81 % of the inspiratory neurones were recorded from the ipsilateral side of the medulla. They were found scattered within the lateral tegmental field in a region that approximates the dorsal respiratory group (Tian et al., 1998c); one was located within the ventral respiratory group. These locations are in general agreement with those found by Ono et al. (1994, 1998) who reported that inspiratory hypoglossal premotor neurones in cats tend to be located dorsomedial to the ventral respiratory group and ventrolateral to the hypoglossal motor nucleus. Unlike Ono et al. (1994, 1998), I found that most of the inspiratory hypoglossal premotor neurones were located dorsal to the ventral respiratory group and ventrolateral to the hypoglossal motor nuclei. This disagreement about the location of hypoglossal premotor neurone may be due to differences in species studied or methodology. Differences are known in dorsal respiratory group neurone function between rats (Tian and Duffin, 1998) and

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cats (Long and Duffin, 1986) as well as in the projections of phrenic premotor neurones (Duffin and van Alphen, 1995a). However, methodological differences may also provide an explanation; Ono et al. (1994, 1998) used spike-triggered averages of extracellular signals to detect functional connexions rather than cross-correlation of discharges. I suggest that upon careful examination, their data (see figure 5E (Ono et al., 1994) and figure 3E (Ono et al., 1998)) support polysynaptic connexions to hypoglossal motoneurones rather than the monosynaptic connexions I found.

Evidence in rats to confirm a location for hypoglossal premotor neurones near the ventral respiratory group is contradictory. Using tract-tracing methods in in-vitro neonatal rat preparations, some investigators have observed inspiratory neurones clustered dorsomedially to the ventral respiratory group that project to the hypoglossal motor nucleus (Wilson et al., 1999). However, Ugolini (1995) found no evidence for such neurones in or around the ventral respiratory group in adult rats. In other tracing experiments, it has been reported that neurones located within the lateral tegmental field project to the hypoglossal motor nucleus in adult rats (Dobbins and Feldman, 1995). They are clustered in two areas and project specifically to hypoglossal motoneurones whose axons project to the lateral and medial branches of the hypoglossal nerve. Despite an extensive search of the lateral tegmental field, I found little evidence for a functional connexion between inspiratory neurones located within the ventral respiratory group and the hypoglossal motoneurones. This observation is consistent with my findings that very few (3.8 %) inspiratory phrenic premotor neurones of the ventral respiratory group excite hypoglossal motoneurones (see Chapter 5) (Peever and Duffin, 2000; Peever et al., 2000). I therefore concluded that in rats the majority of hypoglossal premotor neurones are located diffusely throughout the lateral
 tegmental field, and very few are located within the ventral respiratory group. Although tracing experiments found many neurones located in close proximity to the ventral respiratory group projecting to the hypoglossal motor nucleus, I suggest that they may not transmit respiratory drive but rather non-respiratory drives such as those from brainstem pattern-generators for swallowing, licking or chewing (Amri et al., 1990; Dellow and Lund, 1971; Sumi, 1970; Travers and Jackson, 1992).

While the tongue is not specifically involved in breathing, its activation during inspiration alters airway resistance so as to maintain a patent upper airway (Fregosi and Fuller, 1997; Remmers et al., 1978) and therefore should be synchronised with diaphragmatic contraction. While a dual projection of inspiratory premotor neurones to both hypoglossal and phrenic motor pools would achieve this synchronisation, it would also provide a similar timing with respect to the respiratory cycle, something I did not observe (see Chapter 5). In addition, I found no evidence that hypoglossal premotor neurones also excited phrenic motoneurones; the cross-correlograms computed between the discharges of the 19 hypoglossal premotor neurones and that of the ipsilateral phrenic nerve were featureless. However, in rats and cats, neuroanatomical studies suggest that hypoglossal and phrenic motoneurones receive projections from a common population of neurones located close to or from within the ventral respiratory group (Lipski et al., 1994; Sasaki et al., 1989). In addition, in cats Ono et al. (1994) found functional evidence for such a dual role. Either species difference or differences in the interpretation of data obtained using different methods could account for this disagreement as I previously explained.

I also point out that in contrast to phrenic premotor neurones, which are densely located around and within the ventral respiratory group (Peever and Duffin, 2000; Tian and
Duffin, 1996c), I found the hypoglossal premotor neurones scattered throughout the lateral tegmental field, and that very few (3.8 %) phrenic premotor neurones activate hypoglossal motoneurones (see Chapter 5) (Peever and Duffin, 2000; Peever et al., 2000). Evidence that further supports the independence of phrenic and hypoglossal premotor populations comes from cross-correlograms with repetitive peaks and troughs. These repetitive cross-correlogram features are consistent with those reported by Cohen et al. (1997), who terms them high-frequency oscillations. Although infrequently observed, 10 cross-correlograms with the phrenic nerve, and 1 instance with both phrenic and hypoglossal nerves, these high-frequency oscillations had frequencies similar to those previously reported in rats (134 Hz) (Tian and Duffin, 1996a), but higher frequencies than in cats (50-100 Hz) (Cohen et al., 1987). It is hypothesised that high-frequency oscillations are indicative of synchronising inputs arising from the network of respiratory neurones, which generate and transmit respiratory rhythm (Cohen et al., 1997). If this interpretation is correct, I suggest that hypoglossal and phrenic do not receive synchronising inputs from a common population of inspiratory premotor neurones, reinforcing my view that respiratory rhythm is transmitted to hypoglossal and phrenic motoneurones by separate premotor populations.

6.4.4 Hypoglossal Interneurones and Premotor Neurones: Functional Significance and Conclusions

My observations led me to the following general conclusions about the source of inspiratory drive to hypoglossal motoneurones in rats; there is a small distinct population of inspiratory neurones scattered in the lateral tegmental field that are directly premotor to hypoglossal motoneurones, the source of inspiratory drive is separate from that for phrenic
motoneurones, and the transmission of inspiratory drive may involve hypoglossal interneurones.

The latter conclusion provides an explanation reconciling the disparate observations of numerous anatomical studies that identified projections to hypoglossal motor nucleus from specific locations and my finding of few monosynaptic connexions from scattered locations. Similarly, the lack of shared premotor neurones by hypoglossal and phrenic motoneurones found in my studies might be explained by hypoglossal interneurones interposed in the transmission pathway. The existence of hypoglossal interneurones is supported by my finding neurones located within the hypoglossal motor nucleus (see figure 6.3) that could not be antidromically activated from the hypoglossal nerve, and whose cross-correlograms displayed features that clearly differentiate them from those of hypoglossal motoneurones.

While such observations are the first functional evidence for hypoglossal interneurones in rats, many investigators have speculated about their existence (Boone and Aldes, 1984; Cooper, 1981; Sumi, 1969; Takasu and Hashimoto, 1988; Takata, 1993; Viana et al., 1990). Neuroanatomically, hypoglossal interneurones from cat, rat and monkey have been distinguished from motoneurones by their small size, oval shape and limited dendritic arborisations as compared to the larger, multipolar hypoglossal motoneurones retrogradely labelled from the hypoglossal nerve (Boone and Aldes, 1984; Cooper, 1981; Takasu and Hashimoto, 1988; Takata, 1993). Electrophysiologically, interneurones have been described in intact cats based on their characteristic long latency synaptic potentials following hypoglossal nerve stimulation (Green and Negishi, 1963; Sumi, 1969; Takata, 1993). In the in-vitro slice preparation of guinea-pig brainstem, a population of hypoglossal neurones was distinguished from hypoglossal motoneurones based on their maximal discharge rates (up to
250 Hz vs. 90 Hz) and these authors presumed them to be interneurones (Laursen and Rekling, 1989; Viana et al., 1990). In rats, identified interneurones contact hypoglossal motoneurone dendrites via putative GABAergic terminals (Takasu and Hashimoto, 1988; Takata, 1993).

Although these previous studies identify putative interneurones, no specific evidence has been presented as to their function, but because the tongue participates in different motor acts, hypoglossal interneurones could serve a variety of functions. Based on my data, it might be suggested that hypoglossal interneurones participate in the respiratory control of hypoglossal motoneurones. From the observed cross-correlogram features, I suggest that interneurones receive excitation from respiratory neurones and then monosynaptically inhibit hypoglossal motoneurones. Such an observation is consistent with the anatomical data demonstrating GABAergic interneurones synapsing onto hypoglossal motoneurones, and in agreement with reports that hypoglossal motoneurones receive concomitant excitation and inhibition during inspiration (Withington-Wray et al., 1988; Woch and Kubin, 1995).

A further argument in support of hypoglossal interneurones concerns the function of the tongue. To orchestrate precise tongue movements and position, hypoglossal motoneurones receive synaptic inputs from central pattern generators governing different behaviours (e.g., respiratory, deglutatory, and masticatory) (Lowe, 1981; Ono et al., 1998; Travers and Jackson, 1992). Indeed, numerous neuroanatomical studies, delineate hypoglossal premotor neurones throughout the brainstem, pons and cortex, and attribute this diversity to the tongue’s many functions (Borke et al., 1983; Holstege et al., 1977; Li et al., 1993; Takada et al., 1984; Ugolini, 1995). A complex interneurone organisation would allow finely tuned control of tongue position and movement.


CHAPTER 7

GENERAL DISCUSSION

7.0 OVERVIEW

To understand how motor systems generate and transmit motor commands, it is essential not only to define the components of the system, but also to determine how individual components of the network are interconnected. I applied such an approach to the brainstem respiratory network that generates and transmits the motor commands, which control breathing muscles. In both adult and neonatal rat preparations, I used tissue lesions to characterise the anatomical distribution of the rhythm-generating network, and used highly sophisticated electrophysiological techniques to infer synaptic connexions among its transmission pathways. Specifically, I examined the interconnexions among the medullary respiratory neurones, which transmit respiratory drive to hypoglossal and phrenic motoneurones.

The data presented in this thesis significantly increase our understanding of how the respiratory rhythm-generating network is organised, and how respiratory rhythm is transmitted to spinal and cranial motoneurones. Not only did I characterise the premotor pathways to hypoglossal motoneurones-a novel finding itself, I also provided novel evidence that a functionally identifiable population of hypoglossal interneurones also participate in the control of hypoglossal motoneurones. These data are the first to demonstrate that the pathways and underlying mechanisms of respiratory drive transmission to hypoglossal and phrenic motoneurones differ. In addition, these data demonstrate that the bilateral
connectivity of premotor pathways to respiratory motoneurones in adult rats may not be fully developed in neonatal rat preparations.

In Chapters 3 to 6, I presented my results and discussed them in the context of each chapter. However, in this general discussion, I attempt to synthesise the data to construct a unified theme of how respiratory rhythm is generated and subsequently transmitted to hypoglossal and phrenic motoneurones in the rat. First, I will relate how these data impact our current understanding of mammalian respiratory rhythm generation, and then I will summarise how respiratory rhythm is transmitted to hypoglossal and phrenic motoneurones. Lastly, I will speculate why the respiratory premotor control of hypoglossal and phrenic motoneurones is functionally different, and why hypoglossal interneurones are involved in the respiratory motor control of hypoglossal motoneurones.

7.1 Respiratory Rhythm Generation

7.1.1 Adult Rats

An autonomous network of neurones located throughout the brainstem generates respiratory rhythm (Duffin et al., 1995; Rekling and Feldman, 1998; St.-John, 1998); however, it is unclear which subsets of these neurones are synapticly interconnected to form the “rhythm-generator”. One theory predicts that a population of spontaneously rhythmic pacemaker neurones forms the rhythm-generator (Butera et al., 1999; Koshiya and Smith, 1999b; Rekling and Feldman, 1998), while other theories posit that mutual inhibition between different neuronal populations form bi- or tri-stable oscillators, which ultimately comprise the rhythm-generating kernel (Duffin, 1991; Duffin et al., 1995; Ezure, 1990; Richter et al., 1986). Regardless of mechanisms, it is well agreed that the primary rhythm-
generating network resides within the bilateral columns of the ventral and dorsal respiratory groups (Bianchi et al., 1995; Long and Duffin, 1986). I took advantage of this bilateral distribution to test the hypothesis that each half of the rhythm-generating network can function independent of the other. In adult rats, I found that like the cat, monkey and rabbit (Gromysz and Karczewski, 1981; Gromysz and Karczewski, 1982; Gromysz and Karczewski, 1984; Janczewski and Karczewski, 1984; Karczewski and Gromysz, 1982; Kubin et al., 1987; St.-John, 1983), each side when separated from the other is capable of independently generating and transmitting respiratory rhythm.

Such an observation is physiologically important for three reasons. First, it demonstrates that each half of the medulla contains a fully functional network that is capable of independently generating and transmitting a stable respiratory rhythm. Such anatomical and functional redundancy should therefore permit the network to operate despite unilateral damage (e.g. spinal cord injury or medullary infarcts). Second, because rhythm became asynchronous after a mid-sagittal transection, this indicates that cross-connections between each side are critical for maintaining a synchrony between each of the bilateral rhythm-generators. Precisely how two potentially independent rhythm-generators become phase-locked is difficult to test but is of critical importance in understanding how rhythm is generated in an intact network. Third, because each half of the bilateral rhythm-generator contains the appropriate neuronal circuitry to independently generate and transmit rhythm, then future investigation of the underlying connections and mechanisms of rhythm-generation can be restricted to only half the medulla.
7.1.2 Neonatal Rats

The discovery that *in-vitro* brainstem-spinal cord and brainstem-slice preparations generate respiratory-like rhythms (Smith et al., 1991; Suzue, 1984) led many investigators to use them to examine the cellular and synaptic mechanisms required for respiratory rhythm generation (Ramirez and Richter, 1996). Such an approach has undoubtedly enhanced our understanding of the potential mechanisms of respiratory rhythm generation, however, it has unfortunately failed to explain how specific respiratory neurones within the rhythm-generator "interconnect" to produce rhythm.

Using the same approach as I applied to adult rats, I tested the hypothesis that each half of the neonatal medulla contains a functionally independent rhythm-generator. However, unlike adult rats, mid-sagittal transection of the intact (Janczewski and Aoki, 1997; Janczewski and Aoki, 1998) and isolated (McLean and Remmers, 1994) neonatal medulla resulted in the cessation of respiratory motor output as monitored on either phrenic or hypoglossal nerves. Due to the crude nature of such experiments, one cannot definitively ascertain why this occurs. Because there is copious evidence to suggest that the mechanisms of respiratory rhythm generation undergo developmental modifications (see Chapter 1, section 1.7.3) (Hayashi and Lipski, 1992; Hilaire and Duron, 1999; Ramirez et al., 1996) and due to the observed discrepancy between mid-sagittal lesions in adult and neonatal rats, one might conclude that the underlying mechanisms of rhythm generation or the "interconnectedness" of the rhythm-generator is subject to developmental change. Although these are plausible and logical explanations, I do not consider them likely. Based on the observations in Chapter 4 and from recent observations made by Johnson et al. (2000) and
Del Negro et al. (2000), I contend that, as in adult rats, each half of the neonatal medulla is indeed capable of independently generating respiratory rhythm.

It has been recently observed that putative rhythm-generating pre-Bötzinger complex neurones continue to exhibit respiratory-like activity when placed in culture (Johnson et al., 2000). Similarly, it is reported that pre-Bötzinger complex neurones continue to burst with a respiratory-like rhythm following mid-sagittal transection of the in-vitro neonatal rat transverse brainstem-slice preparation (Del Negro et al., 2000). Together these data strongly indicate that each half of the neonatal rhythm-generator contains the appropriate neuronal circuits to independently generate respiratory rhythm. Based on these observations, I argue that midline separation of the neonatal medulla does not abolish the generation of rhythm; rather it abolishes the expression or transmission of rhythm. This postulate is supported by my proposal that the bilateral premotor pathways, which transmit respiratory drive to hypoglossal and phrenic motoneurones are not fully established in neonatal rats. Hence, severing the midline of the neonatal medulla disrupts premotor pathways thereby eliminating the transmission of rhythm to motoneurones, despite its continued generation in each bilateral rhythm-generator. The issue of respiratory rhythm transmission will be discussed more thoroughly below.

7.1.3 Summary

In both adult and neonatal rat preparations, each half of the bilaterally duplicated medullary respiratory network contains the sufficient neuronal circuitry to independently generate respiratory rhythm.

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7.2 Respiratory Rhythm Transmission

7.2.1 Adult Rats

Not only is the medullary rhythm-generator bilaterally duplicated, so are its transmission pathways. Because respiratory rhythm continued following mid-sagittal medullary transection, this presumably indicates that rhythm-transmitting premotor neurones are bilaterally duplicated. Such a presumption was validated by cross-correlation analysis. I found central peaks in cross-correlograms computed between the inspiratory activities of left and right hypoglossal nerves and of left and right phrenic nerves; this indicates that a common population of premotor neurones projects to and synaptically excites the motoneurones comprising left and right hypoglossal and phrenic nerves, respectively. While such an approach does not delineate the anatomical disruption of premotor inputs to hypoglossal and phrenic motoneurones, it does however suggest a bilateral organisation.

Using both electrophysiological and neuroanatomical techniques, the bilateral premotor drivers of phrenic motoneurones have been identified; in both adult rats and cats, they are located in the rostral ventral respiratory group. In cats, they are bilaterally distributed, but their axons project only to contralateral phrenic motoneurones (Long and Duffin, 1986). In adult rats however, phrenic premotor neurones are bilaterally located and sent bifurcating axons down both sides of the spinal cord, which monosynaptically excite motoneurones in both left and right phrenic motor nuclei (see Chapter 5; Tian et al., 1998a; Tian et al., 1998c).

This thesis provides the first detailed evidence of precisely how respiratory rhythm is transmitted to hypoglossal motoneurones. By cross-correlating the extracellular action potentials of inspiratory neurones of the lateral tegmental field with hypoglossal nerve
discharge, I identified a population of neurones that selectively and monosynaptically project to and excite hypoglossal motoneurones (see figure 7.0). I therefore termed these bilaterally situated neurones, inspiratory hypoglossal premotor motoneurones. Because cross-correlation of their activity with the contralateral hypoglossal nerve produced narrow peaks at short latencies in cross-correlograms, I conclude that not only are these premotor neurones bilaterally duplicated, but they also send axons that excite motoneurones in both left and right hypoglossal motor nuclei. This observation is consistent with the observation that cross-correlation of left and right hypoglossal nerves produced central peaks in cross-correlograms. In addition, I found that cross-correlation of the inspiratory activities of medial and lateral branches of the hypoglossal nerve produced cross-correlograms that displayed narrow central peaks. I interpret this observation to indicate that motoneurones of both medial and lateral nerve branches receive inspiratory drive from a common population of premotor neurones. An observation consistent with the findings of Fuller et al. (1998) and Ryan et al. (2000), who report that tongue retractors and protrusors are synchronously activated during inspiration.

In adult rats, I conclude that the premotor neurones, which project to and synaptically excite hypoglossal and phrenic motoneurones are situated bilaterally, and have axons that bifurcate to excite both left and right motor nuclei. This arrangement therefore ensures that the motoneurones of left-right hypoglossal and phrenic motor nuclei are synchronously excited, so that both sides of the tongue and diaphragm contract together. I also conclude that the motoneurones of medial and lateral hypoglossal nerve branches receive inspiratory drive from the same premotor neurones.
7.2.2. Neonatal Rats

In both intact rats and in-vitro preparations, mid-sagittal transection of the neonatal medulla abolishes the expression of respiratory rhythm (see Chapter 3; Janczewski and Aoki, 1997; McLean and Remmers, 1994). There are two possible explanations to account for this observation. The first possibility is that both halves of the bilateral respiratory network are required to generate respiratory rhythm; however, I do not find this explanation tenable for reasons previously discussed (see section 7.1.2). Alternatively, it is possible that transection of the medulla stops the transmission of respiratory drive to hypoglossal and phrenic motoneurones because the bilateral premotor pathways are not fully developed in neonatal rats; the data from Chapter 4 supports this contention. In both the in-vitro brainstem-spinal cord and transverse brainstem-slice preparations, I found that cross-correlation of the inspiratory activities of left-right hypoglossal nerves and left-right phrenic nerves produced flat cross-correlograms. The lack of cross-correlogram features indicates that neither left-right hypoglossal or phrenic motoneurones receive inspiratory drive from a common population of premotor neurones. Accordingly, I conclude that unlike adults, the bilateral projections of premotor neurones are not fully established in neonates, and propose that contralateral pathways form before the ipsilateral ones do. This would account for both the lack of bilateral synchronisation in left-right hypoglossal and phrenic nerves, and the effects of medullary mid-sagittal transection. Moreover, this would account for observations that mid-sagittal medullary transection abolishes diaphragmatic activity in 2-9 day-old rats but not in 17-22 day-old rats (Janczewski and Aoki, 1997; Janczewski and Aoki, 1998).

Why bilateral premotor projections are not fully established in neonates, I do not
know. There is, however, anecdotal evidence from other maturing motor systems that contralateral premotor pathways develop and predominate before ipsilateral ones (Galea and Darian-Smith, 1995; Lowrie, 1999; Martin J.H. and Lee, 1999; Martin et al., 1999). Despite the lack of bilateral premotor projections to the motoneurones in left-right hypoglossal and phrenic motor nuclei, both left and right sides contract in general synchrony, which must ultimately arise from within the bilaterally synchronized medullary rhythm-generating network.

7.3 RESPIRATORY PREMOTOR CONTROL OF HYPOGLOSSAL AND PHRENIC MOTONEURONES

The respiratory control of spinal motoneurones, specifically phrenic motoneurones, has been thoroughly reported in both adult cat and rat (Ezure, 1990; Long and Duffin, 1986; Monteau and Hilaire, 1991; Tian et al., 1998c). However, the data reported in this thesis are the first to specifically document how respiratory drive is transmitted to the hypoglossal motoneurones, which innervate the tongue in rats. Importantly, these data delineate a separate respiratory control of hypoglossal and spinal motoneurones in adult rats, which provides a significant contribution to understanding the neural control of respiratory muscles.

As for phrenic motoneurones (Long and Duffin, 1986; Monteau and Hilaire, 1991; Tian et al., 1998c), intracellular recording from hypoglossal motoneurones illustrates that they are synaptically excited during inspiration (see Chapter 6). However, unlike phrenic motoneurones, which receive postsynaptic inhibition from Bötzinger complex expiratory neurones (Tian et al., 1998a), hypoglossal motoneurones are not synaptically inhibited by Bötzinger complex expiratory neurones (see figure 7.0). That hypoglossal motoneurones
lack expiratory inhibition is further confirmed by the observation that intracellular injection of chloride current into identified motoneurones did not reverse expiratory hyperpolarization to depolarisation. Accordingly, I contend that hypoglossal motoneurones are disfacilitated during expiration, as they are in adult cats (Withington-Wray et al., 1988; Woch and Kubin, 1995).

Not only is the expiratory control of hypoglossal and phrenic motoneurones different, so is their inspiratory control. I found that very few (3.8 %) of the ventral respiratory group inspiratory neurones that monosynaptically project to and excite phrenic motoneurones also project to and excite hypoglossal motoneurones. Rather, I found that inspiratory neurones located diffusely throughout the lateral tegmental field monosynaptically project to and excite hypoglossal motoneurones; however, these neurones do not also project to and excite phrenic motoneurones (see figure 7.0). This observation contradicts findings in adult cats, which report that inspiratory neurones located within the lateral tegmental field project to and excite both phrenic and hypoglossal motoneurones (Ono et al., 1994; Ono et al., 1998).

7.3.1 Hypoglossal Premotor Neurones: Functional Significance

Although the muscles of the tongue are not specifically involved in breathing, they alter airway resistance so as to maintain a patent upper airway during inspiration (Fregosi and Fuller, 1997; Iscoe, 1988) and therefore should be synchronised with diaphragmatic contraction. While a dual projection of inspiratory premotor neurones to both hypoglossal and phrenic motor pools would achieve this synchronisation, it would also provide a similar timing with respect to the respiratory cycle. This is not necessarily desirable and indeed is not observed. Moreover, the tongue is involved in other motor functions, such as, chewing,
swallowing, suckling, and speaking (Lowe, 1981), and therefore receives synaptic excitation from other central pattern generators (Amri et al., 1990; Ono et al., 1998). It could be argued that the heterogeneity of excitatory inputs necessitates the need for a population of hypoglossal premotor neurones separate from the phrenic premotor neurones, such that each pattern generator synapses upon a specific subset of hypoglossal premotor neurones (Borke et al., 1983; Dobbins and Feldman, 1995). My findings support this view.

Other observations in support of hypoglossal premotor neurones that are separate from those of the phrenic premotor neurones come from the differential responses of the tongue and diaphragm to sleep-state, as well as chemical and pharmacological stimuli. During rapid-eye movement sleep the inspiratory drive to the upper airway decreases significantly more than it does to the diaphragm; a condition potentially leading to obstructive sleep apnoea (Fenik et al., 1998). Hypoglossal and phrenic nerve activities are differentially altered in response to a number of afferent stimuli (Ukabam et al., 1992; Wasicko et al., 1993) including carbon dioxide, with the phrenic nerve being more sensitive than the hypoglossal nerve (Fukuda and Honda, 1982b). Morin et al. (1992) demonstrated that serotonin decreases the discharge amplitude of the hypoglossal nerve more than the phrenic nerve and suggest this observation might be due to different premotor innervation of the two motor nuclei.

As previously mentioned, one of the non-respiratory functions of the tongue is in vocalisation, and a lack of expiratory modulation would serve this function since all forms of phonation are achieved entirely during the expiratory phase. Therefore, lack of inhibition during expiration would permit better control of hypoglossal motoneurones and thereby
tongue muscles because motoneurones would be more easily excited by other non-respiratory premotor inputs.

7.4 **Hypoglossal Interneurones**

One of the most interesting observations in this thesis is the identification of a putative population of interneurones within the hypoglossal motor nucleus. Several lines of evidence support the presence of hypoglossal interneurones. First, there are inspiratory neurones within the hypoglossal motor nucleus that, unlike identified hypoglossal motoneurones, could not be antidromically activated. While such an observation does not verify that non-antidromically activated neurone are indeed interneurones, this has been one of the criterion used to indicate the presence of interneurones within both spinal and cranial motor nuclei (Green and Negishi, 1963; Kirkwood et al., 1988; Sears, 1964a; Sumi, 1969). To further classify these putative hypoglossal interneurones, I cross-correlated their extracellular discharge with that of the ipsilateral hypoglossal nerve to determine their functional relationship with hypoglossal motoneurones. I found that cross-correlograms were of two types: those that displayed broad central peaks alone (52 %); and, those that displayed broad central peaks followed by troughs at short latencies (48 %). Such features are strikingly different from those produced by cross-correlation of hypoglossal motoneurone and nerve, which produced cross-correlograms displaying very narrow peaks at very short latencies.

These inspiratory neurones were classified as hypoglossal interneurones for the following three reason: (1) they are intermingled with identified motoneurones within the stereotaxically defined hypoglossal motor nucleus; (2) unlike motoneurones, they could not
be antidromically activated from the hypoglossal nerve; and most importantly, (3) cross-correlation of their activity with the hypoglossal nerve produced cross-correlograms that were clearly distinct from those for hypoglossal motoneurones.

Based primarily upon neuroanatomical observations, the presence of hypoglossal interneurones has been suggested previously (Takata, 1993; Viana et al., 1990). Because hypoglossal motoneurones are recruited to activate tongue muscles that participate in various behavioural acts, their premotor control is undoubtedly complex. Accordingly, it has been proposed that interneurones might function to ‘finely tune’ the control of hypoglossal motoneurones (Boone and Aldes, 1984; Takasu and Hashimoto, 1988; Takata, 1993). My data are the first to show that hypoglossal interneurones are involved in the respiratory-related control of hypoglossal motoneurones. This claim is supported not only by the fact that these neurones display inspiratory-related discharge patterns, but moreover, by the observation that their discharge is tightly correlated with that of hypoglossal motoneurones. Although these observations do not describe how interneurones participate in transmitting respiratory drive to hypoglossal motoneurones, they nonetheless implicate their involvement in respiratory control.

By interpretation of cross-correlogram features, I put forth several hypotheses of how hypoglossal interneurones might transmit respiratory drive to hypoglossal motoneurones. Since all cross-correlograms computed between hypoglossal interneurones and motoneurones displayed broad central peaks, I suggest that both receive inspiratory drive from a common premotor population. The candidate premotor source would be presumably from the lateral tegmental field inspiratory neurones, which monosynaptically project to and excite hypoglossal motoneurones (figure 7.0).
Although all cross-correlograms displayed broad central peaks, some also displayed troughs shortly after, which may indicate that, not only are there hypoglossal interneurones, there may be two types of them. Those interneurones that are commonly activated and exhibit central peaks in their cross-correlograms with the hypoglossal nerve discharge; and those that are inhibitory and produce central peaks followed by troughs, which indicate an inhibition of hypoglossal motoneurones. The basis for the latter interpretation comes from two lines of evidence. First, combined neuroanatomical and immunohistochemical data reveal the presence of neurones within the hypoglossal motor nucleus that are separable from motoneurones because of their smaller size and oval shape, and because they are GABAergic (Takasu and Hashimoto, 1988; Takata, 1993). Second, I used a recently developed cross-correlation simulation program (Duffin, 2000) to imitate the effects two neurones receiving a common excitation while one neurone inhibits the other; such a simulation produced cross-correlograms that were remarkably similar to the ones I observed (see figure 6.11). Based on this evidence, I therefore suggest that inhibitory hypoglossal interneurones are excited during inspiration so that they inhibit hypoglossal motoneurones during this phase.

How hypoglossal interneurones participate in the transmission of respiratory rhythm to hypoglossal motoneurones is currently unclear. However, because the tongue is involved in many different motor functions, it would seem intuitive that a ‘higher’ level of premotor control would be advantageous. Accordingly, I propose that hypoglossal interneurones act as a “gate” to control hypoglossal motoneurones.

The existence of interneurones within the hypoglossal motor nucleus has wide ranging implications ranging from the manner by which sleep networks impinge on hypoglossal motoneurones during different sleep-states (Horner, 1996; Yamuy et al., 1999),
to the interpretation of hypoglossal activity in transverse brainstem-slice preparations. Further exploration of hypoglossal interneurones needs careful attention in order to understand how the respiratory control system and other non-respiratory behaviours are coordinated to enable precise control of tongue movements and position.

7.5 SUMMARY

Figure 7.0 illustrates how respiratory rhythm is transmitted to hypoglossal and phrenic motoneurones in the rat. In summary, I made the following conclusions:

(1) In both adult and neonatal rats, the respiratory rhythm-generating network is bilaterally duplicated.

(2) In adult rats, the inspiratory premotor neurones, which transmit respiratory rhythm to phrenic and hypoglossal motoneurones are bilaterally duplicated and send axonal projections to both left and right hypoglossal and phrenic motoneurones. In neonatal rats, the bilateral axonal projections of inspiratory premotor neurones are not fully established.

(3) The ventral respiratory group premotor neurones, which transmit inspiratory and expiratory drives to phrenic motoneurones, do not transmit it to hypoglossal motoneurones. The respiratory control of phrenic and hypoglossal motoneurones is from separate premotor populations.

(4) Hypoglossal motoneurones receive inspiratory drive from premotor neurones located diffusely throughout the lateral tegmental field. Hypoglossal motoneurones are not actively inhibited during expiration; they are disfacilitated.
(5) Interneurones within the hypoglossal motor nucleus may also participate in the transmission of respiratory rhythm to hypoglossal motoneurones in adult rats.
Figure 7.0. A schematic representation of the premotor connexions to hypoglossal (XII) and phrenic motoneurones in adult rats. Inspiratory drive is transmitted to phrenic motoneurones by inspiratory premotor neurones in the ventral respiratory group (VRG-I); and expiratory drive is transmitted to phrenic motoneurones by inhibitory Bötzinger complex expiratory neurones. The majority of respiratory drive is transmitted to XII motoneurones by inspiratory premotor neurones located within the lateral tegmental field (LTF). Interneurones within the XII motor nucleus may also be involved in the transmission of respiratory drive to XII motoneurones.
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