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**Development of upper respiratory tract motor nuclei in the rat
(*Rattus norvegicus*)**

Friedland, David Richard, Ph.D.

City University of New York, 1993

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**DEVELOPMENT OF UPPER RESPIRATORY TRACT
MOTOR NUCLEI IN THE RAT (*RATTUS NORVEGICUS*)**

by

DAVID RICHARD FRIEDLAND

**A dissertation submitted to the Graduate Faculty in Biomedical Sciences in
partial fulfillment of the requirements for the degree of Doctor of Philosophy,
The City University of New York**

1993

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This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract**DEVELOPMENT OF UPPER RESPIRATORY TRACT
MOTOR NUCLEI IN THE RAT (*RATTUS NORVEGICUS*)****by****DAVID RICHARD FRIEDLAND****Advisors: Jeffrey T. Laitman, Ph.D.
Avrim R. Eden, M.D.**

The mammalian upper respiratory tract serves as the common modality for functions of respiration, deglutition and vocalization. The role of the URT in establishing these functions is largely defined by muscles of the tongue, pharynx and larynx. As such, the neuromuscular system subserving URT control must be capable of coordinating activity between these muscle groups and of eliciting actions specific to individual URT behaviors. Little is known, however, regarding the development of URT neuromuscular control, in particular, of the brainstem motor nuclei innervating URT muscles.

This study investigated the development of URT motor nuclei innervating muscles of the tongue, pharynx and larynx. Specifically, the course of motoneuron cell death in the nucleus ambiguus (NA) and the hypoglossal nucleus (XII) was charted in pre- and postnatal rats. Motoneuron cell death (MCD) is a normal developmental process by which an initial overproduction of motoneurons is reduced to adult levels in response to epigenetic signals from target muscles and afferent neurons. The development of NA and XII was further studied by experiments charting migration of URT motoneurons and the establishment of GABAergic innervation in both nuclei.

Results indicated MCD in both URT motor nuclei occurring entirely prenatally. MCD in the hypoglossal nucleus (XII) appeared to initiate approximately one day before that

in the nucleus ambiguus (NA). Further, cell death in XII consisted of a loss of 40-45% of motoneurons versus 55-60% in NA. Migration and interneuron studies supported the methodologies used to assess MCD. In addition, these studies indicated that NA motoneurons arise from two regions of the basal plate and that GABAergic innervation was established in NA and XII during the period of MCD.

This investigation indicates the presence of critical periods in the development of URT motor nuclei during late gestation in the rat. As such, a template has been established upon which to predicate the development of normal and pathological URT neuromuscular control in humans. Discussions have been included with regard to URT incoordination pathologies such as the Sudden Infant Death Syndrome, and the role of MCD in the evolution of the human URT and the origins of articulate speech.

**THIS DISSERTATION IS DEDICATED TO THE MANY PARENTS
FACED WITH UNANSWERED QUESTIONS WITH THE HOPE THAT,
THROUGH SCIENCE, WE MAY SOMEDAY UNDERSTAND AND
PREVENT WHAT IS NOW A MYSTERY AND A TRAGEDY**

ACKNOWLEDGEMENTS

I would like to express my deepest appreciation and gratitude to my advisors Dr. Jeffrey T. Laitman and Dr. Avrim R. Eden. I wish to thank Dr. Laitman for his intimate involvement in all aspects of this project and my graduate training, particularly: teaching me the wonders and excitement of gross, developmental, evolutionary and comparative anatomy; providing the guidance and opportunities for me to develop my own teaching and presentation skills; correcting and refining the text of this dissertation and all abstracts written over the past years; and for all his support, encouragement and enthusiasm throughout my training. I would like to thank Dr. Eden for his generosity in providing me valuable laboratory space, all the equipment and supplies necessary for these investigations, opportunities for travel and presentation of my work, continued contact with the clinical aspects of my training, and encouragement and support in all my studies. I would like to thank both my advisors for recognizing my "independent streak" and allowing me to forge and define my own project and giving me the freedom in the laboratory to explore many pathways and methodologies for completing this study. Further, they have become colleagues and friends who have welcomed me into their lives, their homes and their families enriching my life and for which I am gratefully and eternally indebted.

I would like to thank the members of my graduate and dissertation committees: Dr. Paul Lazarow, for the support of the Department of Cell Biology and Anatomy, and for his personal integrity with regard to precision and accuracy in science which has greatly improved the quality of this thesis; Dr. Victor Friedrich, for his enthusiasm and expertise in all aspects of this study, particularly, his help with statistical analyses and fluorescent microscopy; Dr. Marilyn McGinnis, for her expertise in rat neurobiology and her help in preparing this document and in coordinating the graduate program during my training; Dr. John Morrison, for his help, and the help of his laboratory, in the immunocytochemical aspects of this project; Dr. Ian Tattersall, for his generosity in teaching and for helping place

this work in an evolutionary context; and Dr. Thomas Van De Water, for his unending enthusiasm and helpful suggestions, particularly with regard to characterization of prenatal animals and the use of fast-DiI.

I also wish to express my gratitude to Dr. Hugh Biller for the generous support of the Department of Otolaryngology, for providing my first introduction to head and neck surgery, and as a role model for integrity and honesty in Medicine. Within the department I would like to thank Dr. Ira Sanders for the use of laboratory equipment, particularly the vibratome, which was essential to this study. I wish to thank Dr. Margaret Brandwein for her assistance in histology and URT development. In addition, I wish to thank Lisa Carey for her assistance throughout this study, particularly with regard to photography. Special thanks to Shirley Singh for all her help in obtaining supplies and equipment and in filing and completing the myriad of forms necessary for this study. In addition, I wish to thank Yasmin Azeez, Florence Lafayette and Sylvia Giamarino of Otolaryngology, and Minnie Woodson of Cell Biology and Anatomy for their support and friendship over the years.

I would like to express my sincerest appreciation and thanks to Mr. Patrick Gannon. He has been exceedingly generous in spending countless hours showing me laboratory techniques critical to all investigations in this study. Further, he has shared with me his knowledge of neurobiological and developmental issues which were the bases of many specific features of the experimental designs. I would also like to thank Patrick for his loyal friendship over the past years and for welcoming me into his home and life graciously.

I would also like to thank Dr. Wen-Xiang Zhang, who as instructor of microvascular surgery for the Departments of Surgery and Otolaryngology, has spent many hours teaching me the proper techniques, methods and equipment necessary for quality microdissection and microsurgery. His steady hands and guidance were essential to the prenatal fast-DiI and GABAergic studies.

Many discussions I have had regarding aspects of this study have proved invaluable to my thesis. I wish to thank Dr. Joy Reidenberg for our discussions of rat development and

for providing photographs for use in this dissertation. I also would like to thank Dr. Wan-Hua Amy Yu for our several conversations regarding the anatomy of the rat hypoglossal nucleus and aspects of cell counting. I would also like to thank Dr. Robert Baker for his instruction on the use of DiI and fast-DiI.

The companionship and friendship of many individuals helped make the graduate experience an enjoyable one and kept me motivated and directed. I wish to thank my friends in the program who have provided empathetic ears and have always been supportive: Yasodha Natkunam, Waldo Feng, Josina Reddy and Susie Snyder. I also wish to thank Maria Rivera for her never-ending charm and enthusiasm. I would also like to thank Youzhu Li and Liancai Mu for their humor and friendship. Further, I wish to thank the many residents who have passed through the lab the past four years for their friendship and support.

I would particularly like to thank my parents for their constant support and love in all I have endeavored. I want to thank my father, Shelly, and mother, Anita, for always giving me the emotional, financial and physical support I've needed in my non-academic life, so that I could whole-heartedly concentrate on my academic studies. I also want to thank my brother, Michael, for his support and love in all aspects of my academic and personal life.

Finally, I wish to thank my wife and best-friend, Karin, who has always been supportive and understanding of all I have done. Her encouragement and enthusiasm have helped me through the "lows" and made the "highs" that much brighter. As we face our own unanswered questions may we become even closer and approach the future as one, with courage and hope.

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Chapter 1

Introduction and Scope of Research; and an Introduction to the Anatomy and Neurology of the Upper Respiratory Tract

1.1 Introduction and Scope of Research

The overall goal of this research has been to understand the coordination of developmental processes between central neuronal control centers and peripheral muscular components of the mammalian upper respiratory tract (URT). The specific aim has been to characterize the prenatal development of brainstem motor nuclei subserving URT function. In particular, the course of motoneuron cell death (MCD), a naturally occurring degenerative process in developing motor systems, was charted in the nucleus ambiguus and the hypoglossal nucleus of the rat. These data have been used to establish critical periods in the formation of URT neuromuscular control, and to define a baseline pattern of MCD in mammalian brainstem motor nuclei upon which development in the specialized human upper respiratory system may be predicated.

This project has addressed the anatomy, physiology, and maturation of the peripheral URT as they influence, and may coordinate with, the development of central neuronal systems. The occurrence of MCD has been used as a vehicle for understanding developmental periods critical to the establishment of normal postnatal URT function. While the factors which influence the course of MCD in general are of great intrinsic importance, it has not been the goal of this study to focus solely upon such mechanisms.

As this project has endeavored to understand coordinated development between brainstem neuronal centers and components of the peripheral URT, it has been necessary to gain an appreciation of both regions so that the above noted neuronal changes could be appreciated fully. Accordingly, this thesis has been organized to present discussions of the peripheral upper respiratory region and the related control divisions of the brainstem, in addition to material on MCD.

Chapter 1 will discuss aspects of URT anatomy, development and innervation; chapter 2, the development of the brainstem, and neuroanatomy and physiology of the URT motor nuclei; and chapter 3, the discovery, characterization, and mechanisms of

motoneuron cell death. These background chapters will be followed by chapter 4 which focuses on the rationale and choice of the rat as the experimental model; and details both the URT and central brainstem anatomy of the rat. Chapter 5 will describe the methods used in the investigation of MCD in this study. Chapters 6 and 7 will describe the investigation of MCD in the nucleus ambiguus and hypoglossal nucleus, respectively. Chapter 8 will describe the methods used in the investigation of nucleus formation and neuronal characterization. Chapters 9 and 10 will detail the investigation of such phenomena in the nucleus ambiguus and hypoglossal nucleus, respectively. Chapter 11 will contain conclusions and discussions regarding all results; and implications from this study for understanding both the evolution of neuromuscular control of the mammalian URT and the etiology of neuromuscular incoordination pathologies during human development.

1.2 Introduction to the Upper Respiratory Tract

The upper respiratory tract (URT) consists of the nasal and oral cavities, the pharynx, connections to and including the middle ear, and constituent structures such as the larynx and tongue. In most mammals, particularly humans, the region serves as the common modality for functions of respiration, deglutition, and vocalization. Accordingly, the region is often described by various terms including the upper digestive tract, the aerodigestive tract, and the vocal tract. The term "upper respiratory" will be used here as it is the classically used term and describes the primary function of the region (Crelin, 1976; Laitman, 1977). The URT is the "crossroads" of the mammalian pathways of respiration, deglutition and vocalization and, as such, plays a critical role in the health and survival of the organism.

While the URT is a region comprised of many elements, including important skeletal and cartilaginous components (Laitman and Crelin, 1976; Laitman et.al, 1978;

Grossmangin, 1979; Laitman and Reidenberg, 1988; Reidenberg and Laitman, 1991) its role in establishing patterns of respiration, deglutition and vocalization is largely defined by the soft-tissue structures. Specifically, the muscles of the tongue, pharynx and larynx play critical roles in determining the course of URT functions. As such, the neuromuscular system subserving URT control must be capable of coordinating activity between these muscle groups and of eliciting behaviors specific to individual URT functions. Indeed, incoordination of neuromuscular control may be a major underlying etiology of many upper respiratory, upper digestive and vocal tract disorders (Sasaki and Isaacson, 1988; Logemann, 1988; Shapiro and Healy, 1988). The following sections will discuss the anatomy, function, development and innervation of the URT, with particular emphasis on the muscles and their neural control.

1.3 The Mammalian Upper Respiratory Tract

The anatomical relationship between structures of the URT, as found in situ, has been used to define an overall URT morphology. A major feature of this morphology has been shown to be laryngeal position in the neck (Negus, 1949; Laitman, 1977; Reidenberg, 1988; Laitman and Reidenberg, 1993a, 1993b). Specifically, observations on the relationship between the epiglottis, the supero-anterior most cartilage of the larynx, and the soft palate have defined a baseline mammalian URT morphology. This is characterized by a larynx positioned high in the neck with the epiglottis able to overlap the soft palate. This creates what has frequently been referred to as the "intranarial" position of the epiglottis or larynx (Howes, 1889; Negus, 1949; Laitman, 1977).

The baseline mammalian URT morphology creates largely separate pathways for respiration and deglutition. Indeed, studies based on both dissection of postmortem material, as well as those using radiographs, cineradiographs, and CT scans have shown that most mammals may have the ability to breathe and swallow many foods almost

simultaneously due to these distinct routes (Laitman, 1977; Laitman et.al., 1977; Sasaki et.al., 1977; Laitman and Crelin, 1980a; Reidenberg and Laitman, 1990; Flugel and Rohen, 1991; German and Crompton, 1993).

Comparative anatomical studies in the laboratory of Laitman and colleagues, in particular, have demonstrated the existence of high laryngeal position across a wide array of aquatic and terrestrial mammals (figure 1.1) (e.g., Laitman, 1977; Laitman and Crelin, 1980a; Reidenberg and Laitman, 1987; Reidenberg and Laitman, 1990). While variations in level of the larynx can be found the overall high position allows for laryngeal "inter-locking" and the creation of two largely separate pathways.

The basic mammalian URT morphology is also found in fetal and early postnatal humans. Recent studies have shown the fetal period to be an intense time of maturation of laryngeal position and overall URT morphology (figure 1.2, a, b). During this time frame, aspects of the larynx are gradually developing and by the 23-25th week have been shown to be sufficiently developed to allow for epiglottic-soft palate contact (Magriples and Laitman, 1987). Indeed this time frame appears to be important for the maturation of the entire upper respiratory region including portions of the skull base (Magriples and Laitman, 1987; Wolfson and Laitman, 1990; Isaacson and Birnholz, 1991; Moore and Laitman, 1991). Perinatal and early postnatal periods also exhibit high laryngeal position with the possibility of epiglottic-soft palate contact (figure 1.2, c-e) (Crelin, 1973; Sasaki et.al., 1977; Laitman and Crelin, 1980b). Thus, newborn humans have largely distinct pathways for respiration and deglutition and, like other mammals, can suckle and breathe almost simultaneously. However, during the first several years of life, the larynx gradually descends in the neck enlarging the oropharynx and making opposition of the epiglottis and soft palate impossible (figure 1.2, f)(Roche and Barkla, 1965; Laitman and Crelin, 1976; Crelin, 1976; Flugel and Rohen, 1991; Laitman and Reidenberg, 1993b). Thus, the mature human is distinct from other mammals, as well as its own newborns and early infants, in the positional relationships of its URT.

Figure 1.1: Midsagittal sections through the URT of the common dolphin (a; *Delphinus delphis*), harbor seal (b; *Phoca vitulina*), domestic pig (c; *Sus scrofa*), sheep (d; *Ovis aries*), cat (e; *Felis domestica*), and olive baboon (f; *Papio anubis*), (E: epiglottis; S: soft-palate; T: thyroid cartilage of larynx; C: cricoid cartilage of larynx). Note that the epiglottis opposes the soft-palate. This is considered an "intranarial", or high-positioned, larynx and defines the baseline mammalian URT morphology. (Figure from Laitman and Reidenberg, 1993a, with permission of the authors)

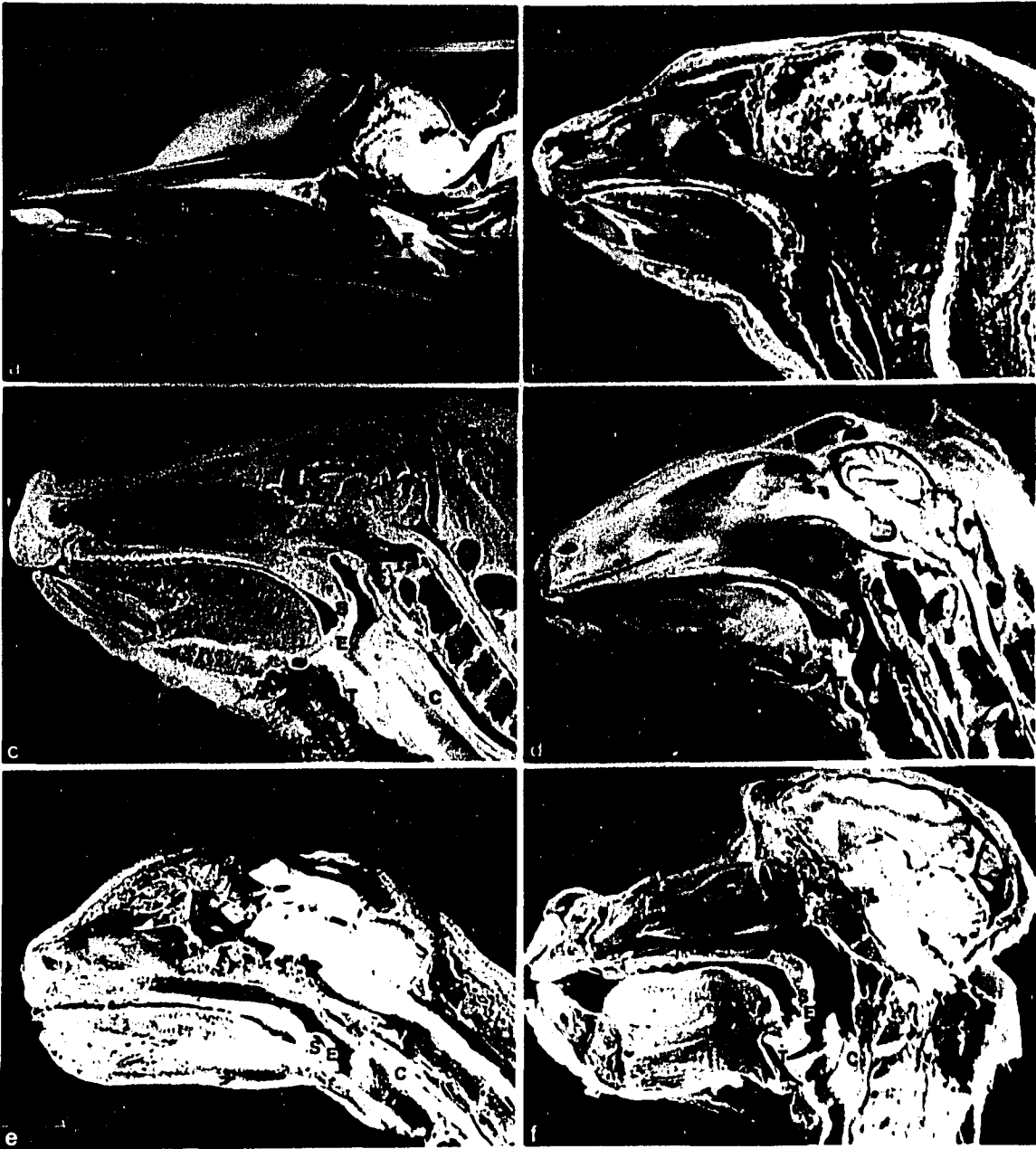


Figure 1.2: Midsagittal sections through the URT of the human at various prenatal and postnatal ages (a, 15 week fetus; b, 24 week fetus; c, newborn; d, 7 months; e, 15 months; f, adult), (E: epiglottis; S: soft-palate; T: thyroid cartilage of larynx; C: cricoid cartilage of larynx). Note that prenatal and early postnatal humans exhibit a baseline mammalian URT morphology with the possibility of epiglottic and soft-palate opposition. In contrast, the adult human larynx has descended in the neck creating an enlarged oropharynx in which pathways for respiration and deglutition cross. (Figure from Laitman and Reidenberg, 1993a, with permission of the authors)



The unique structure of the adult human URT has altered the physiologic and functional relationships found in organisms exhibiting an intranasal larynx. That is, the descent of the larynx has created an enlarged oropharyngeal space which serves as a common pathway for inspired air and ingested foods. Accordingly, adult humans are more dependent upon precise neuromuscular control to separate functionally tracts which were previously anatomically distinct. For example, among the functional demands placed on the human URT neuromuscular system is protection of the airway during swallowing and maintenance of pharyngeal and glottic patency during inspiration (Sasaki and Isaacson, 1988; Logeman et.al., 1992). However, such dependency carries with it an increased risk of potentially lethal upper airway obstructions (Heimlich and Uhley, 1979; Feinberg and Ekberg, 1990; Laitman and Reidenberg, 1993b). The advantage, however, of a low larynx is that it creates an enlarged oropharynx capable of increased modification of initial laryngeal sounds (Laitman, 1983; Laitman, 1984; Crelin, 1987; Lieberman et.al., 1992).

1.4 Gross Anatomy

By acting in a coordinated and precise fashion, muscles of the URT evoke the complex neuromuscular actions necessary for proper patterns of respiration, deglutition, and vocalization. While many muscles are involved in URT control, this study will focus upon those of the oral cavity and pharynx (i.e., tongue and pharyngeal muscles) and the intrinsic muscles of the larynx. The following sections will provide a brief overview of these structures, and define the peripheral anatomical substrate to which the following chapters on central neuroanatomy will relate. For further, more detailed discussions on the anatomy, physiology, development and pathology of the region see Negus (1949, 1965), Fink (1975), Laitman, (1977), Bailey and Biller (1985), Bailey (1993).

1.4.1 The Larynx and Laryngeal Muscles

The larynx is a musclocartilaginous structure located at the proximal end of the trachea. It has evolved within vertebrates as a protective mechanism to guard the trachea and lungs from foreign material (Negus, 1949; Sasaki and Isaacson, 1988; Kirchner, 1991). The following anatomical descriptions are based largely upon the human condition and discussions of inter-species variation can be found in Negus (1949) and Wind (1970). The larynx, unlike the pharynx and tongue, has a skeletal framework comprised of nine main cartilages (three unpaired, three paired). The largest is the thyroid, so named for the Greek "thyreos" or shield, as it guards the front of the throat. It is a semi-circular structure, open posteriorly, composed of wide, flat laminae fused in the midline. In humans, the anterior-superior surface of the thyroid cartilage exhibits a characteristic notch and the laryngeal prominence which is often pronounced in males (the so called "Adam's apple"). Posteriorly, the thyroid cartilage exhibits two horns or cornua. The superior cornu projects rostrally to associate with the greater horn of the hyoid via the thyrohyoid ligament. The inferior cornu articulates with another laryngeal cartilage, the cricoid. The cricoid cartilage (from the Greek "krikos" meaning ring), is a complete ring, thicker than the thyroid, and about 2-3 cm wide posteriorly. Situated superiorly, on the posterior aspect of the cricoid cartilage, are the paired arytenoid cartilages and their associated corniculate cartilages. From the arytenoid cartilages arise the aryepiglottic folds which contain the small cuneiform cartilages and attach superiorly to the epiglottis. The epiglottis is a flat, fibrocartilaginous structure which projects superiorly along the anterior border of the laryngeal and pharyngeal lumen. As described above, in all mammals and newborn humans this structure opposes the soft palate to "lock" the larynx into the nasal cavity. In adult humans, however, the epiglottis projects no higher than the posterior border of the base of the tongue.

The internal aspect of the larynx is formed by sheets of broad fibroelastic membranes. The upper portion, often referred to as the quadrangular membrane,

terminates in a free margin known as the vestibular ligament (vestibular folds; also known as the false vocal cords). The lower portion of the membrane (cricothyroid membrane or cricovocal ligament) terminates in a similar free-edged margin known as the vocal ligament or vocal folds (the true vocal cords). The level of the vocal folds is frequently referred to as the glottis. All positional relationships within the larynx are relative to this point (e.g., supraglottic, infraglottic). These folds, particularly the vocal folds, have evolved in mammals as the primary protective mechanism of the airways. Their coordinated movements and any pathologies which affect them are of concern in all aspects of breathing, swallowing, and vocal behaviors (Bless and Abbs, 1983; Titze and Scherer, 1983).

The majority of laryngeal muscles are those which directly affect movements of the vocal folds and glottic aperture (two pairs of small secondary muscles, the aryepiglotticus and thyroepiglotticus, are involved in movements of the epiglottis). As the vocal folds span between the arytenoid cartilages posteriorly and the thyroid cartilage anteriorly, laryngeal muscles are primarily concerned with altering the position of these cartilages to effect glottic changes. The arytenoid cartilages rotate upon the cricoid, at the synovial crico-arytenoid joint, to result in the majority of abduction and adduction of the vocal folds. As the larynx evolved as a sphincter designed to protect the airway, most intrinsic laryngeal muscles function to adduct, or close the vocal folds. Indeed, laryngeal adductors have been shown to have among the fastest contraction times of any muscles recorded (Sasaki and Isaacson, 1988). Laryngeal adductor muscles include the lateral cricoarytenoids, the transverse arytenoids, the thyroarytenoids, and the oblique arytenoids. In addition, the cricothyroid muscles may cause some narrowing of the glottic aperture although their primary action is to lengthen the vocal folds. Only one muscle functions to abduct (i.e., open) the vocal folds, the posterior cricoarytenoid. However, simultaneous contraction of the posterior cricoarytenoid and cricothyroid muscles can further increase the glottic aperture by concurrent abduction and lengthening

of the vocal folds (Sasaki and Isaacson, 1988; Megirian and Sherrey, 1991; Patrickson et.al., 1991). In respiration, both these muscles are active during inspiration, while the adductors are active during expiration (Green and Neil, 1955; Bartlett, 1986). This response accommodates changes in URT pressures during the respiratory cycle and thus maintains airway patency. Precise movements of glottic musculature are also of considerable importance in studies of phonation and voice quality (Hirose and Gay, 1972; Fujimura, 1977).

1.4.2 The Pharynx and Pharyngeal Muscles

The pharynx is a cylindrical space bounded posterolaterally by muscles attaching to the base of the skull and anteriorly by the internal nares above and posterior tongue below, continuing inferiorly to the upper esophagus (see reviews, King, 1952, Astley, 1958; Bosma and Fletcher, 1961; Bailey, 1993). In adult humans, the pharynx has classically been divided into three regions: the nasopharynx, oropharynx, and laryngopharynx (also frequently termed in clinical literature the epipharynx, mesopharynx, and hypopharynx). The nasopharynx spans rostrally from the internal nares to the lower border of the soft palate caudally; the oropharynx from the soft palate to the epiglottic tip inferiorly and to the palatoglossal arch anteriorly; the laryngopharynx from the epiglottic tip to the beginning of the upper portion of the esophagus at the lower level of the cricoid cartilage.

The walls of the pharynx are largely muscular, consisting of circularly arranged fibers of the paired pharyngeal constrictors and, in adult humans, the posterior portion of the tongue. The superior constrictor arises from the pterygoid hamulus, pterygomandibular raphe, and deep surface of the mandibular ramus before attaching to the pharyngeal tubercle of the occipital bone; the middle constrictor attaches anteriorly to the greater and lesser horns of the hyoid and posteriorly to the fibrous pharyngeal raphe; the inferior constrictor attaches anteriorly to the cricoid and thyroid cartilages and

posteriorly to the raphe. The lower border of the inferior constrictor, often known as the cricopharyngeus muscle, has a major role in functioning as part of an upper esophageal sphincter. The pharyngeal constrictors, along with the tongue, are the major muscles involved in movements of a food bolus from the oral cavity through the pharynx to the esophagus (e.g., Hiiemae and Crompton, 1985; Palmer et.al., 1992). The constrictors have also been shown to be active during expiration (Sherrey and Megirian, 1975; Bartlett, 1986). In addition to the constrictors, three pairs of small longitudinal muscles function in movements of the pharyngeal wall. These are the stylopharyngeus, palatopharyngeus, and salpingopharyngeus muscles. The stylopharyngeus arises from the styloid process and passes inferiorly to blend with the middle constrictor; the palatopharyngeus arises from the soft palate and similarly blends with the pharyngeal coat; the salpingopharyngeus arises from the cartilage of the pharyngotympanic (Eustachian or auditory) tube and runs inferiorly to blend with the palatopharyngeus. These muscles are capable of elevating the pharynx and function principally during deglutition.

1.4.3 The Tongue and Lingual Muscles

The tongue is the principle structure of the oral cavity and, in adult humans, comprises the anterior wall of the pharynx (see reviews Sonntag, 1925; Abd-el-Malek, 1939; Doran, 1975; Lowe, 1981). Two types of muscles comprise the tongue: extrinsic and intrinsic. Those muscles arising from bone and projecting into the body of the tongue are considered extrinsic. These include the genioglossus, hyoglossus, palatoglossus, and styloglossus. Those muscles having little, if any, bony attachments and found entirely within the body of the tongue are called intrinsic. These include the superior and inferior longitudinal muscles, the transversus and the verticalis. Of these, the inferior longitudinalis may have some direct attachments to the hyoid bone while the others are wholly intrinsic. There has been disagreement concerning the functions of

these two groups of tongue muscles. Some have believed that intrinsic and extrinsic muscles functioned as largely independent units (e.g., Whillis, 1946) while others have emphasized that muscles from both groups act together, in a coordinated fashion, to elicit all lingual movements (Abd-el-Malek, 1938; Lowe, 1981).

In addition to the above lingual muscles, the geniohyoid, a suprahyoid muscle, is closely associated with protrusion of the tongue, working with the genioglossus to maintain airway patency during inspiration (Bartlett, 1986). It is also closely related to other lingual muscles by virtue of the central location of its innervating motoneurons (see chapter 2). The palatoglossus is classically thought of as more of a palatal muscle as it is not considered to play a major role in movement of the tongue (Williams et.al., 1989). In addition, its distinct innervation from the other lingual muscles suggest a different evolutionary origin (Sokoloff and Deacon, 1992).

1.5 Embryology

One of the most prominent features in prenatal development is the segmentation of the embryo (classically defined as the period of organogenesis, in humans conception to eight weeks) into mesodermal blocks of tissue called somites. This segmentation establishes readily predictable patterns of innervation in the mature organism. For example, early somite segmentation of the embryo results in the formation of dermatomes, regularly repeated fields of neural innervation. In contrast, many structures of the head and neck are not derived from somites and their innervation patterns are not so predictable. They are, however, formed from distinct condensations of mesenchymal tissue known as the branchial arches (Kingsbury, 1926).

The branchial arches (so named due to their resemblance to the "branchia" or gills in fish) begin to form in a rostral-caudal direction shortly after the appearance of the first cervical somites. The arches are separated externally by a pharyngeal cleft and internally

by a pharyngeal pouch. Each arch has a specific cartilaginous and muscular component, and a specific artery and cranial nerve. While the muscles of the head and neck often migrate to their mature locations, they retain the specific cranial nerve innervation established in the branchial arches. Components of the pharynx and larynx are derived from three branchial arches and are therefore innervated by the three respective cranial nerves. The tongue is largely derived from somites, yet has some branchial arch components (e.g., palatoglossus muscles), and its innervation is reflective of this process. The following sections will discuss the branchial arches, their derivatives and the embryologic formation of URT structures. Additional reviews of head and neck embryology can be found in Frazer (1910), Kingsbury (1926), Tucker and O'Rahilly (1972), Paparella and Shumrick (1973), O'Rahilly and Tucker (1973), Crelin (1976) and O'Rahilly and Muller (1984).

1.5.1 Branchial Arches and Their Derivatives

1.5.1.1 First Branchial Arch

The first branchial arch contributes in large part to the facial and cranial skeleton. The cartilaginous components define the maxillary and mandibular processes. The latter is often called Meckel's cartilage. These cartilages almost completely regress in the mature organism forming only the heads of the malleus and incus. The mesenchyme of these processes, however, through membranous ossification forms the mandible, maxilla, zygomatic bone, and most of the temporal bone.

The muscular derivatives of the first arch consist of the muscles of mastication (masseter, temporalis, lateral and medial pterygoids), the anterior belly of the digastric, the mylohyoid, the tensor tympani, and the tensor veli palatini. All these muscles are innervated by the nerve of the first arch, the trigeminal nerve, also known as the fifth cranial nerve (CN V). Specifically, the third division of the trigeminal nerve, the mandibular division, supplies all motor fibers to the muscular derivatives of the first

branchial arch. Sensory innervation of the face is by all three divisions of the trigeminal nerve; the ophthalmic, the maxillary, and the mandibular. In addition, the trigeminal supplies sensory fibers to the mucosa of the anterior 2/3 of the tongue. This is due to the fact that the coverings of the anterior tongue develop from the first arch. The muscles of the tongue, however, arise from occipital myoblasts that migrate through the first arch.

The separations between the arches, the clefts and pouches also give rise to specific structures. The first pharyngeal cleft gives rise to the external auditory meatus and part of the tympanic membrane. The first pharyngeal pouch forms the narrow tubotympanic recess which forms a sac at its distal end. The sac becomes the middle ear cavity and part of the tympanic membrane. The narrow portion of the recess persists as the pharyngotympanic tube connecting the nasopharynx with the middle ear.

1.5.1.2 Second Branchial Arch

The cartilage of the second arch is called Reichert's cartilage. It forms the inferior parts of the incus and malleus, and all but the footplate of the stapes. The second arch cartilage also forms the styloid process and the lesser horns of the hyoid bone.

Muscular derivatives of the second arch are muscles of facial expression, the posterior belly of the digastric, the stylohyoid, the stapedius, and auricular muscles. These muscles are innervated by the nerve of the second arch, the facial nerve, also known as the seventh cranial nerve (CN VII). The facial nerve also contributes sensory fibers to the skin around the external ear and taste fibers to the anterior 2/3 of the tongue. These taste fibers are carried in a branch of CN VII known as the chorda tympani. This branch communicates between the first and second arches and is thought to represent the pretrematic branch of the second branchial arch nerve. Trema, is the term for the space between branchial arches and the pretrematic nerve traverses this space to innervate components of the preceding branchial arch. Thus, the facial supplies sensory fibers to the second arch and part of the first arch, specifically, the surface of the tongue.

The second pharyngeal pouch enters the surrounding mesenchyme which is later invaded by mesodermal tissue. These form the anlagen of the palatine tonsils which are infiltrated with lymphatic tissue during fetal development. The pouch remains in the mature organism as the tonsillar bed or fossa. The second pharyngeal cleft disappears as the second arch proliferates and grows over the third and fourth arches. Persistence of the second pharyngeal cleft results in a lateral cervical cyst which communicates with the surface via the branchial fistula.

1.5.1.3 Third Branchial Arch

The third branchial arch has few cartilaginous and muscular derivatives. It forms the greater horn and inferior body of the hyoid bone and only one muscle, the stylopharyngeus. This muscle is supplied by the nerve of the third arch, the glossopharyngeal nerve, the ninth cranial nerve (CN IX). Via its motor control of the stylopharyngeus, and extensive sensory contribution to the URT, CN IX works in close conjunction with the nerves of the fourth through sixth branchial arches (see below). As its name implies the nerve supplies both, regions of the pharynx and the tongue, providing sensory and taste fibers to the covering of the tongue, caudal to the foramen caecum. The coverings of the posterior tongue derive from the second and third arches, the latter arch overgrowing the former. Thus, the non-muscular components of the posterior tongue are of branchiomic origin and supplied by the glossopharyngeal nerve. The glossopharyngeal also supplies sensory fibers to skin around the external ear.

The third pharyngeal pouch, along with the fourth, has a unique winged shape. The ventral wing forms the thymus gland and the dorsal wing the inferior parathyroids. The thymus migrates toward the anterior thorax and carries with it the parathyroids. The parathyroids remain in the neck dorsal to the thyroid gland in the mature organism.

1.5.1.4 Fourth Through Sixth Branchial Arches

The terminologies and distinctions of the fourth through sixth branchial arches are not as clear as the first three. Indeed, the existence and or derivatives of the fifth branchial arch remains controversial and the distinctiveness of components of the fourth and sixth branchial arches is also often unclear. These branchial arches are thus often considered together as they collectively contribute to the walls of the pharynx and the structure and muscles of the larynx. The laryngeal skeleton is formed by the fusion of derivatives of these arches including the thyroid, cricoid, arytenoid, corniculate, and cuneiform cartilages. The epiglottis appears to be a unique mammalian addition which may not have been part of the original branchial arch system (Laitman and Reidenberg, 1993).

All the muscles of the larynx appear to be derived from the sixth arch except the cricothyroid muscle which originates from the fourth arch. The nerve of the fourth arch is the vagus nerve, the tenth cranial nerve (CN X) while the nerve of the sixth (possibly fifth as well) is usually described as the cranial root of the spinal accessory nerve, part of the eleventh cranial nerve (CN XI). However, the cranial root of the spinal accessory nerve is distributed with the vagus and thus the entire larynx may be considered to be innervated by branches of the tenth nerve (see following for discussion). As the vagus is the nerve of the fourth arch it also supplies the other muscular derivatives such as the levator palatini and portions of the pharyngeal constrictor muscles.

As mentioned above, the fourth pharyngeal pouch has a winged appearance. The dorsal wing forms the superior parathyroid glands which attach to the dorsal surface of the migrating thyroid gland. The ventral wing forms a small amount of thymus which soon disappears. An additional outpocketing of the fourth pouch is often considered the fifth pharyngeal pouch. It gives rise to the ultimobranchial body which forms the parafollicular cells of the thyroid gland. The fourth and fifth pharyngeal clefts, like the third, are overgrown by the second arch and normally disappear.

The fourth through sixth branchial arches join together to form the pharynx and larynx. In addition, the third arch contributes to the structure of the pharynx via the stylopharyngeus muscle. Thus, a large part of the URT is derived from separate embryologic entities each with distinct neural innervations. As such, normal URT control in the postnatal organism is dependent upon coordinated development between the arches and their respective cranial nerves. It is therefore not surprising that the motoneurons of the glossopharyngeal, vagus, and cranial root of the spinal accessory are all found in the same region of the brainstem, the nucleus ambiguus. This will be discussed in chapter 2.

1.5.2 URT Non-branchial Arch Derivatives

Aspects of the development of the tongue were briefly described as it related to branchial arch coverings. The muscles of the tongue (with the exception of the palatoglossus), however, arise from occipital somites. Myoblasts from these somites migrate from alongside the medulla into the developing tongue. This non-branchiomic origin of tongue muscles determines that the innervation of the tongue will similarly arise from non-branchiomic nerves. The majority of motor innervation of the tongue is carried in the hypoglossal nerve, the twelfth cranial nerve (CN XII). Thus, the URT is formed from three branchial arches and several occipital somites representative of four motor nerves whose motoneurons are in distinct brainstem nuclei.

1.6 Neuroanatomy

While the branching of nerves to the URT often appears complex due, for example, to the overriding of contiguous branchial arch territories, the central brainstem organization of their respective nuclei is relatively ordered. This is due to the organization of the lower brainstem into columns of cells consisting, largely, of the same

fiber type. For example, all special visceral afferent (SVA) fibers have their terminal fields in the nucleus of the solitary tract although they are associated with the facial, glossopharyngeal, and vagus nerves. This section will focus on the nerves innervating the URT, their major branches, fiber types, and brainstem origins. These descriptions are based primarily on the human condition as they have been the most extensively studied. For further descriptions see Williams et.al. (1989), Boles (1973), Cummings et.al. (1986) and for non-human primates Sprague (1944).

The majority of nerves innervating the URT are a subset of the "cranial" nerves (some muscles of the URT, e.g., infrahyoids, are innervated by branches of the cervical plexus). Aside from formal names (e.g., the olfactory nerve), cranial nerves are numbered from rostral to caudal (e.g., cranial nerve I (CN I)). It is standard usage today to number twelve cranial nerves, however, it should be noted that this numbering is due to tradition and does not necessarily relate to functional reality. Indeed, the recognized number of cranial nerves has varied considerably over the years (see review, Shaw, 1992).

Branches of four cranial nerves innervate the URT: the trigeminal, the glossopharyngeal, the vagus, and the hypoglossal (the cranial root of the spinal accessory is distributed by the vagus). The trigeminal nerve, innervates URT muscles associated with the pharyngotympanic tube, palatal function, or tympanic membrane movement. As the important phenomenon of tubal function and concomitant middle ear aeration is a highly specialized subset of upper airway activity it will not be focused upon here. The understanding of middle ear aeration and tubal function, however, is a major feature of normal mammalian upper respiratory physiology and thus the focus of considerable current investigation (Eden, 1981; Eden and Gannon, 1987; Gannon et.al., 1990; Eden et.al., 1990; Eden et.al., 1992). The glossopharyngeal, vagus, and hypoglossal nerves innervate muscles of the tongue, pharynx, larynx, and soft palate.

1.6.1 The Glossopharyngeal Nerve: Cranial Nerve IX

1.6.1.1 Anatomy

The glossopharyngeal nerve is a mixed nerve, containing both sensory and motor fibers. A large percentage of its efferent fibers innervate salivary glands and mucous membranes. As noted, the glossopharyngeal nerve innervates only one muscle, the only muscular derivative of the third branchial arch, the stylopharyngeus. The nerve is formed by the rostral most of a series of rootlets that contribute to the vagal and spinal accessory nerves as well. These leave the brainstem caudal to the pons just dorsal to the inferior olive. After union of these rootlets in the region of the jugular fossa, the superior (jugular) and inferior (petrous) glossopharyngeal ganglia are found. These contain the cell bodies of sensory neurons which form a major part of the glossopharyngeal nerve. The ganglia are located in the jugular foramen through which CN IX exits the skull.

1.6.1.2 URT Branches

Pharyngeal Branches. As the glossopharyngeal nerve passes along the middle constrictor it gives off several branches which join pharyngeal fibers of the vagus and laryngotracheal fibers of the sympathetic trunk. These various fibers form the pharyngeal plexus through which the glossopharyngeal nerve supplies sensory fibers to the mucosa of the pharynx.

Muscular Branch. As the glossopharyngeal nerve passes along the posterior border of the stylopharyngeus muscle it gives off a motor branch to the muscle. This is the only component of the glossopharyngeal nerve supplying efferent fibers to a muscular derivative of the third branchial arch.

Tonsillar Branches. After supplying a motor branch to the stylopharyngeus muscle the glossopharyngeal nerve passes between the superior and middle constrictor muscles to form a plexus around the tonsils supplying sensory fibers to the mucosal membranes of the palate and faucial tonsils.

Lingual Branches. In addition to supplying the tonsillar plexus the glossopharyngeal nerve gives off two lingual branches. The anterior branch supplies the mucus membranes of the tongue and vallate papillae in the region of the sulcus terminalis. The other branch communicates with the lingual nerve to supply taste to the posterior one third of the tongue.

1.6.1.3 Fiber Types: Central Origins and Terminations

GSA: Spinal Nucleus of the Trigeminal Nerve These fibers carry general sensation from skin around the external ear, mucosa of the middle ear, inner surface of the tympanic membrane, and upper pharynx. The cell bodies of GSA neurons are located in the glossopharyngeal ganglia (petrous and jugular) and the fibers terminate centrally in the spinal nucleus of the trigeminal tract. These fibers are found in the tympanic, tonsillar, lingual, and pharyngeal branches.

GVA: Nucleus of the Solitary Tract Neurons conveying sensation from the carotid bodies, posterior third of the tongue, and possibly middle ear chemo/baroreceptors (Gannon et.al., 1990) have their cell bodies in the inferior or jugular glossopharyngeal ganglion. Their fibers terminate in the dorsal part of the nucleus of the solitary tract after traveling caudally through the solitary tract itself. GVA fibers are found in the carotid sinus, tympanic, and lingual branches of the glossopharyngeal nerve.

SVA: Nucleus of the Solitary Tract Neurons conveying taste from the vallate papillae and posterior third of the tongue also have their cell bodies in the inferior glossopharyngeal ganglion and fibers which terminate in the nucleus of the solitary tract. However, unlike fibers from the carotid sinus, fibers for taste terminate in the ventral portion of the nucleus. Taste is conveyed by the lingual branches to the tongue.

GVE: Inferior Salivatory Nucleus Innervation of the glands of the head and neck is via the superior and inferior salivatory nuclei. The superior nucleus distributes with the facial nerve while the inferior nucleus by the glossopharyngeal nerve. The inferior

salivatory nucleus contains preganglionic parasympathetic fibers which distribute through the tympanic and lesser petrosal nerves to the otic ganglion. Here, fibers synapse with postganglionic parasympathetic fibers which distribute with the auriculotemporal branch of the trigeminal nerve to the parotid gland and minor salivary glands of the mouth and pharynx.

SVE: The Nucleus Ambiguus Motor neurons to the stylopharyngeus muscle are found in the rostral part of the compact formation of the nucleus ambiguus (see chapter 2). Motoneurons from this part of the nucleus ambiguus convey their axons through the main trunk of the glossopharyngeal nerve to leave through the muscular branch to the stylopharyngeus.

1.6.2 The Vagus Nerve: Cranial Nerve X

1.6.2.1 Anatomy

The vagus nerve derives its name from the Latin for wandering. It is so considered due to its extensive innervation throughout the head, neck, thorax and abdomen. The nerve is formed by eight to ten of the same series of rootlets which contribute to the glossopharyngeal nerve. The vagus emerges caudal to the glossopharyngeal and, in similar fashion, contains two sensory ganglia. The proximal most is called the superior vagal, or jugular ganglion, which has a connecting branch to the inferior ganglion of the glossopharyngeal nerve. The inferior, or nodose, ganglion is larger and like the superior contains cell bodies of SVA, GVA, and GSA fibers.

1.6.2.2 URT Branches

Pharyngeal Branch. The pharyngeal branch emerges from the inferior vagal ganglion, passes between the internal and external carotids and pierces the pharyngeal wall at the upper border of the middle constrictor muscle. Here it contributes to the pharyngeal plexus joining fibers from the glossopharyngeal, external laryngeal, and

sympathetic nerves. The plexus innervates muscles of the pharynx and soft palate, except the tensor veli palatini. In addition, it supplies the mucosa of the same structures.

Superior Laryngeal Nerve. The superior laryngeal nerve emerges from the middle of the inferior ganglion and passes external to the pharyngeal muscles toward the thyrohyoid membrane. Here the nerve branches into the internal and external laryngeal nerves. The internal branch is primarily sensory and pierces the thyrohyoid membrane to supply the larynx and pharynx above the vocal folds. In addition, a small number of parasympathetic fibers supply glands of the epiglottis and base of tongue. The external branch passes posteriorly outside the larynx and then pierces the inferior constrictor to supply the cricothyroid muscle and portions of the inferior constrictor.

Recurrent Laryngeal Nerve. The left and right recurrent laryngeal nerves take different pathways to innervate the larynx. The right recurrent nerve branches off the vagus anterior to the right subclavian artery and passes below and behind the vessel as it recurs superiorly. On the left, the nerve branches in the thorax as the vagus passes the arch of the aorta. It loops medially and superiorly around the ligamentum arteriosum to follow the trachea cranially. Both nerves travel in the tracheoesophageal groove and pass underneath the inferior border of the cricopharyngeus muscle to enter the larynx through the cricothyroid membrane. Several branches are given off by the recurrent laryngeal nerves as they course cranially. These are cardiac branches contributing to the cardiac plexus, tracheal and esophageal branches to the muscular and mucosal layers of these structures, pharyngeal branches to the cricopharyngeus (inferior constrictor), and inferior laryngeal branches to the intrinsic laryngeal muscles. The recurrent laryngeal nerves supply all the intrinsic muscles of the larynx except the cricothyroid and supply sensory fibers to the mucosal membranes below the vocal folds. In addition, there are communicating branches between the recurrences and the internal laryngeal nerves superiorly.

Esophageal Branches. These arise superiorly and inferiorly from the recurrenents or the vagal trunks. Superior branches were discussed above and innervate the upper third striated esophageal musculature and the striated parts of the middle third. The inferior branches form the esophageal plexus and are primarily preganglionic parasympathetics supplying smooth muscle in the lower two thirds of the esophagus.

1.6.2.3 Fiber Types: Central Origins and Terminations

GSA: Spinal Nucleus of the Trigeminal Nerve In addition to the trigeminal, facial, and glossopharyngeal nerves, the vagus nerve also contributes to general cutaneous sensation at the external ear and auditory meatus. These fibers are found in the auricular branch of the vagus and project centrally to the spinal nucleus of the trigeminal tract.

GVA: Nucleus of the Solitary Tract The vagus nerve supplies afferent innervation to the majority of thoracic and abdominal viscera. These neurons have their cell bodies in the jugular and nodose ganglia and their fibers terminate in the dorsal portion of the nucleus of the solitary tract. Interestingly, it has been noted that some visceral afferents may terminate in the dorsal motor nucleus of the vagus in rodents (Paxinos, 1985).

SVA: Nucleus of the Solitary Tract The vagus conveys taste fibers to the ventral part of the nucleus of the solitary tract. These fibers travel in the superior laryngeal nerve from the epiglottis and base of tongue. The sensory components and central afferent projections of the vagus are the same as in the glossopharyngeal nerve. These similarities indicate common embryonic origins and imply a coordination of developmental phenomena in the ninth and tenth cranial nerves.

GVE: Dorsal Motor Nucleus of the Vagus Unlike the parasympathetic component of the glossopharyngeal nerve, the vagus nerve supplies no glands in the head and neck. Rather, the vagus supplies autonomic innervation to the thoracic and abdominal viscera. This difference in distribution is reflected in the central origins of these efferent neurons. Whereas glossopharyngeal parasympathetics arose from the inferior salivatory nucleus,

vagal parasympathetics arise from the dorsal motor nucleus of the vagus. In addition, some studies have demonstrated that vagal parasympathetics to the heart and abdominal viscera may arise from the nucleus ambiguus (see chapter 2).

SVE: The Nucleus Ambiguus The motoneurons supplying derivatives of the fourth through sixth branchial arches arise from the nucleus ambiguus. These motoneurons supply the pharynx and larynx through the pharyngeal plexus and the superior and recurrent laryngeal nerves. As mentioned, this nucleus also contains the motoneurons to the stylopharyngeus muscle that travel through the glossopharyngeal nerve.

1.6.3 The Spinal Accessory Nerve: Cranial Nerve XI

1.6.3.1 Anatomy

The spinal accessory nerve, unlike the vagus and glossopharyngeal nerves, is purely a motor nerve innervating both branchiomeric and somatic musculature. These two distinct distributions are reflected in the central locations of spinal accessory motoneurons. Branchiomeric innervation derives from the cranial root and somatic innervation from the spinal root.

Cranial Root The cranial root of the spinal accessory originates as a series of rootlets continuous, but caudal, to those contributing to the vagal and glossopharyngeal nerves. These rootlets join with fibers of the spinal root and pass through the jugular foramen. After leaving the cranium the spinal and cranial roots separate and the cranial root fibers join with the vagus nerve. The cranial root of the spinal accessory distributes to muscles of the larynx and soft palate through the pharyngeal plexus and recurrent laryngeal branches of the vagus.

Spinal Root The spinal root of the spinal accessory originates from superior cervical regions of the spinal cord. Spinal root fibers leave the spinal cord between ventral and dorsal roots and form a trunk which ascends through the foramen magnum. Within the cranium the spinal root joins the cranial root and passes through the jugular foramen.

After separating from the cranial root, the spinal accessory nerve descends medial to the styloid process to pierce the deep surface of the sternocleidomastoid muscle. The nerve supplies the muscle and emerges at its posterior border to cross the posterior triangle of the neck. About 5 cm above the clavicle, the nerve pierces the anterior surface of the trapezius to join with cervical ventral rami. Together these nerves form a plexus which innervates the trapezius muscle.

1.6.3.2 Fiber Types: Central Origins and Terminations

SVE: *The Nucleus Ambiguus* The cranial root of the spinal accessory nerve innervates muscles of branchiomeric origin. As such, its motoneurons are derived from the SVE column of cells in the brainstem, the nucleus ambiguus. These motoneurons are continuous with those of the vagus and glossopharyngeal nerves underlying their common embryonic origins and motor distributions to muscles of the URT.

GSE: *The Spinal Nucleus* The spinal root of CN XI innervates the sternocleidomastoid and trapezius muscles. Whether these fibers should be considered as GSE, or SVE as above, remains somewhat controversial (McKenzie,, 1955; Cihak, 1974). While the central motoneurons are a caudal continuation of the SVE motor column, they are located distal to the foramen magnum and are thus spinal in origin. Further, the embryonic origin of the trapezius and sternocleidomastoid appear to be via somites rather than of branchial arch origin.

1.6.4 The Hypoglossal Nerve: Cranial Nerve XII

1.6.4.1 Anatomy

The hypoglossal nerve is formed by a series of 10-15 rootlets which leave the brainstem caudal and ventromedial to those of the vagus and glossopharyngeal. These rootlets pass through the hypoglossal canal where they join to form a single nerve. Like the spinal accessory, the hypoglossal nerve is considered to contain only motor fibers,

however, electrophysiologic studies have indicated the presence of afferent fibers along its course (see chapter 2). In addition, the hypoglossal nerve is intimately associated with motoneurons of the upper cervical spinal cord, distributing their axons to hyoid muscles via branches of the ansa cervicalis.

1.6.4.2 URT Branches

The hypoglossal nerve does not branch until it reaches the inferior surface of the tongue. Here, it bifurcates into medial and lateral divisions (Abd-el-Malek, 1938) which innervate tongue protrusor and retrusor muscles, respectively (Uemura-Sumi et.al., 1981). Traditionally, components of the first cervical nerve are thought to travel along with the hypoglossal and depart from it before the body of the nerve enters into the tongue. These C1 fibers, often referred to as the descendens hypoglossi (or superior root of the ansa cervicalis) innervate suprahyoid muscles. Interestingly, while the above is the condition classically cited for humans (Williams et.al, 1989), studies on an array of animals, including monkeys and rodents, indicate that the innervation of certain suprahyoid muscles, such as the geniohyoid, may be directly via the hypoglossal nerve itself (Uemura-Sumi et.al., 1981; Sokoloff and Deacon, 1992)

1.6.4.3 Fiber Types: Central Origins and Terminations

GSE: The Hypoglossal Nucleus Motoneurons supplying non-branchiomic muscles of the tongue arise from the hypoglossal nucleus. Neurons projecting through the lateral division of the hypoglossal nerve are found in the dorsolateral region of this nucleus, while those projecting through the medial division are in the ventromedial region. The hypoglossal nucleus is discussed in detail in chapter 2.

Chapter 2

URT Motor Nuclei: The Nucleus Ambiguus and Hypoglossal Nucleus

2.1 Introduction

Central nervous system control of the mammalian URT resides in the brainstem. Specifically, from distinct regions of the medulla, motoneurons send their axons through cranial nerves IX, X, XI and XII to muscles of the larynx, pharynx, and tongue. The two regions of the brainstem in which URT motoneurons are found are called the nucleus ambiguus and the hypoglossal nucleus. The nucleus ambiguus (NA) contains motoneurons which send their axons through the glossopharyngeal and vagus nerves to muscles of the larynx and pharynx. NA also contains motoneurons of the cranial root of the spinal accessory nerve which are distributed through the vagus and recurrent laryngeal nerves. The hypoglossal nucleus (XII) contains motoneurons which send their axons through the hypoglossal nerve to innervate the intrinsic and extrinsic muscles of the tongue, including the geniohyoid muscle.

The URT motor nuclei, and the neuronal centers with which they are integrated, are crucial for the maintenance and control of the upper aerodigestive tract. However, little is known of the prenatal development of URT motor nuclei and the events which allow the establishment of specific connections with central neuronal and peripheral muscular structures. This study seeks to define these developmental events by examining changes in the motoneurons within NA and XII. As such, this chapter will provide the background necessary for the pursuit of this investigation.

In particular, this chapter will describe the general embryology of the brainstem so as to understand the origin and prenatal location of URT motoneurons. Further, each nucleus will be thoroughly described with respect to: 1) brainstem location, so as to define anatomic landmarks for identifying the nucleus; 2) types of neurons in the nucleus, so as to distinguish the development of URT-related motoneurons from non-URT-related cells; 3) organization of neurons within the nucleus with respect to peripheral innervation (somatotopic organization), so as to define the precise locations of

specific URT motoneurons to understand the development of integrated and coordinated function among peripheral URT muscles; 4) peripheral afferent input to the nucleus, so as to define peripheral signals which may influence motoneuron development and the establishment of neuromuscular specificity; 5) central afferent input to the nucleus, so as to define central neuronal signals which may influence URT motoneuron development and the establishment of coordinated patterns of respiration, deglutition, and vocalization.

2.2 Embryology of the Brainstem

Orofacial and URT structures derive from segmented regions of the embryonic head and neck called the branchial arches. Similarly, the cranial motor nuclei projecting to these structures arise in discrete segments of the embryonic brainstem called rhombomeres (see review by Lumsden, 1990). In the vertebrate, there are eight rhombomeres designated r1 through r8 which recent studies have shown are determined by homeobox containing genes of the Hox clusters (Wilkinson et.al., 1989; Wilkinson and Krumlauf, 1990; Hunt and Krumlauf, 1991; Hunt et.al., 1991). This genetically controlled segmentation restricts the formation of brainstem motor systems to specific locations along the rostral-caudal axis. For example, cranial nerves V, VII, and IX project from alternate rhombomeres starting with r2 (Lumsden and Keynes, 1989; Lumsden, 1990). That is, the trigeminal nerve projects from r2, the facial nerve from r4, and the glossopharyngeal nerve from r6. The vagus, spinal accessory, and hypoglossal nerves do not follow this alternating pattern but are associated with r7 and r8. Neural tracing studies have shown that the motoneurons of the fifth, seventh and ninth nerves arise, initially from their corresponding rhombomeres, and subsequently from the immediately caudal segment (Lumsden and Keynes, 1989). As such, rhombomeres r6/r7 and r7/r8 give rise to motoneurons of the glossopharyngeal and vagal/spinal accessory

nerves, respectively, while motoneurons to the hypoglossal nerve arise from r8, exclusively. Thus, the nucleus ambiguus is formed from motoneurons generated in segments r6/r7/r8, and the hypoglossal nucleus, from motoneurons arising solely in r8.

Within rhombomeres, developing neurons are further organized with regard to function and fiber type. For example, neurons arising dorsolaterally are induced to become sensory neurons while those arising medially become motoneurons (Yamada et.al., 1991). The dorsolateral region is called the alar plate and the medial region, the basal plate (figure 2.1). Motoneurons at the basal plate are arranged in three columns which, from medial to lateral, contain general somatic motoneurons, special visceral motoneurons, and general visceral motoneurons (Smith and Williams, 1984; Sadler, 1985). While this is the generally accepted pattern of brainstem neurogenesis, several studies have indicated that the special visceral motoneurons may arise more medially, in the same column as the somatic motoneurons (Kimmel, 1940; Windle, 1970; Brown, 1990). In the medulla, the special visceral motoneurons migrate laterally and ventrolaterally to form the nucleus ambiguus while the general somatic motoneurons remain in the dorsomedial brainstem as the hypoglossal nucleus. This migratory pattern is shown in figure 2.1.

2.3 Nucleus Ambiguus

2.3.1 Introduction

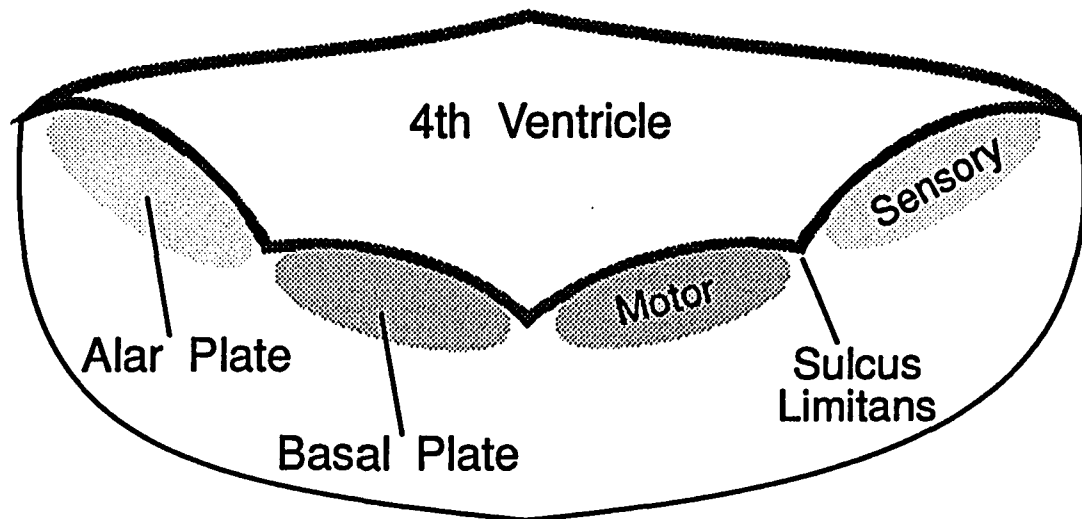
The nucleus ambiguus (NA) is a column of cells located in the ventrolateral brainstem. It contains motoneurons which project through the vagus, glossopharyngeal and spinal accessory nerves to muscles of the larynx, pharynx, tongue (palatoglossus) and soft palate (levator palati) (Furstenberg and Magielski, 1955). The rostral and caudal extents of the nucleus ambiguus (NA) are roughly from the caudal end of the facial nucleus to the pyramidal decussation (Bystrycka and Nail, 1985). On transverse section,

Figure 2.1: Overview of embryologic origins of brainstem motoneurons. The upper figure shows the partitioning of the brainstem into the basal and alar plates. The lower figure shows the partitioning of the basal plate into general visceral efferent (GVE), special visceral efferent (SVE) and general somatic efferent (GSE) cell columns. The SVE and GSE columns are shown overlapping as there is disagreement as to the exact location of their origins (see text for further details). The arrow on the right indicates the path of migration of NA motoneurons innervating the pharynx and larynx to the mature location.

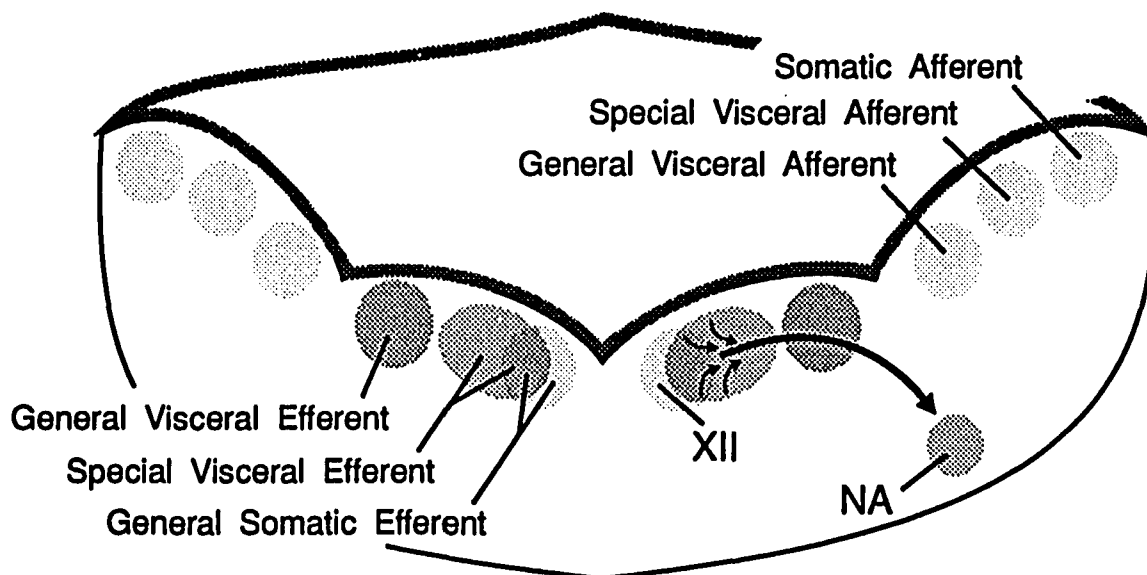
Figure 2.1

Embryology of Brainstem Motor Nuclei

Partitioning of Motor and Sensory Neurons



Partitioning of Neurons By Fiber Type Migration of NA Motoneurons



NA lies in the ventral part of the reticular formation, just dorsal to the lateral reticular nucleus (figure 2.2). Other landmarks have been used and minor variations exist as to the locations of the extreme poles of NA (see review in Yoshida et.al., 1984)

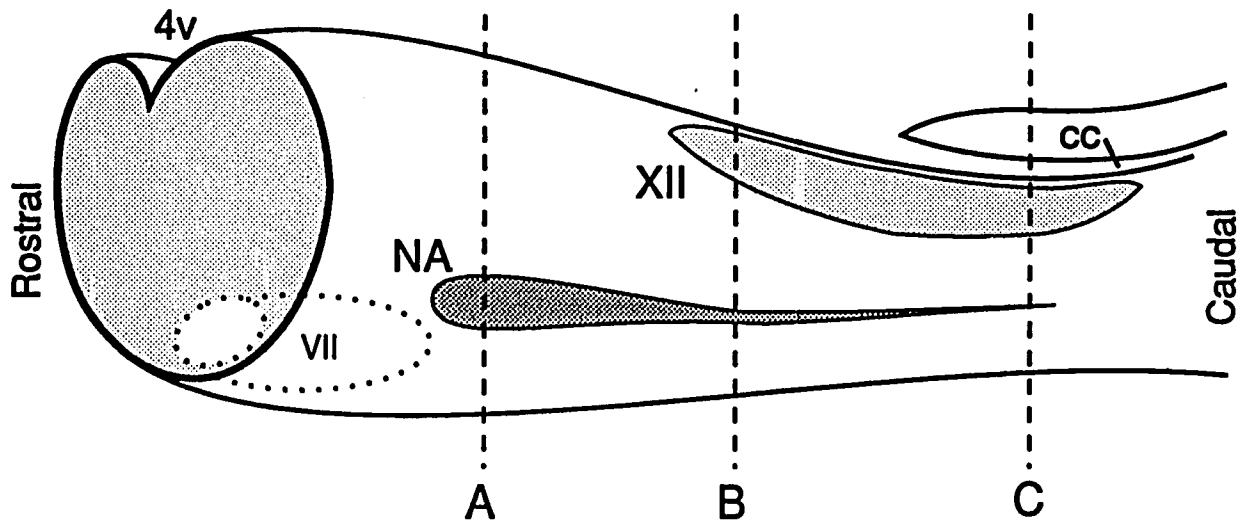
The various descriptions of the rostral limit of NA appear to be more a result of differences in nomenclature than of differences in histological appearance. In 1961, Taber published observations on the cytoarchitecture of the cat brainstem. In this paper she described the rostral pole of NA as being comprised of distinct ventral and dorsal columns. Taber referred to the ventral column as the retrofacial nucleus, a term used by Jacobsohn in 1909 to describe the human NA (cited in Brown, 1990). The use of this nomenclature has persisted in studies of the cat NA, although some researchers have observed no distinct separation between the retrofacial nucleus and the dorsal column of NA (Yoshida et.al., 1982; Holstege et.al., 1983). In addition, several researchers have considered the cat retrofacial nucleus, not as a separate component, but as the rostral-most part of NA (Pásaro et.al., 1983; Van Loveren et.al., 1985). Studies in other species, for example, the rabbit, rat, bat, and monkey, likewise have not described a separate retrofacial nucleus, instead they have referred to a continuous column of cells (Lawn, 1966b; Armstrong et.al., 1983; Yoshida et.al., 1984; Okubo et.al., 1987; Bieger and Hopkins, 1987; Kitamura and Ogata, 1990; Nuñez-Abades et.al., 1992). As such, the retrofacial nucleus can be considered a part of NA comprising a subset of the neurons at the rostral pole. Aside from the retrofacial nucleus, Taber (1961) also defined a nucleus at the caudal end of NA, the retroambiguus nucleus. Studies have not shown this region to be a distinct entity and it has been considered the caudal "tail" of NA (Takayama et.al., 1982; Kalia and Sullivan, 1982; Pásaro et.al., 1983; Bystrycka and Nail, 1985; Bieger and Hopkins, 1987).

Figure 2.2: Schematic representation of a sagittal (top) and transverse (bottom) section through the brainstem. NA is found in the ventrolateral brainstem extending from the facial nucleus to the pyramidal decussation. XII is found in a dorsomedial location and begins caudal to NA, also extending to the decussation.

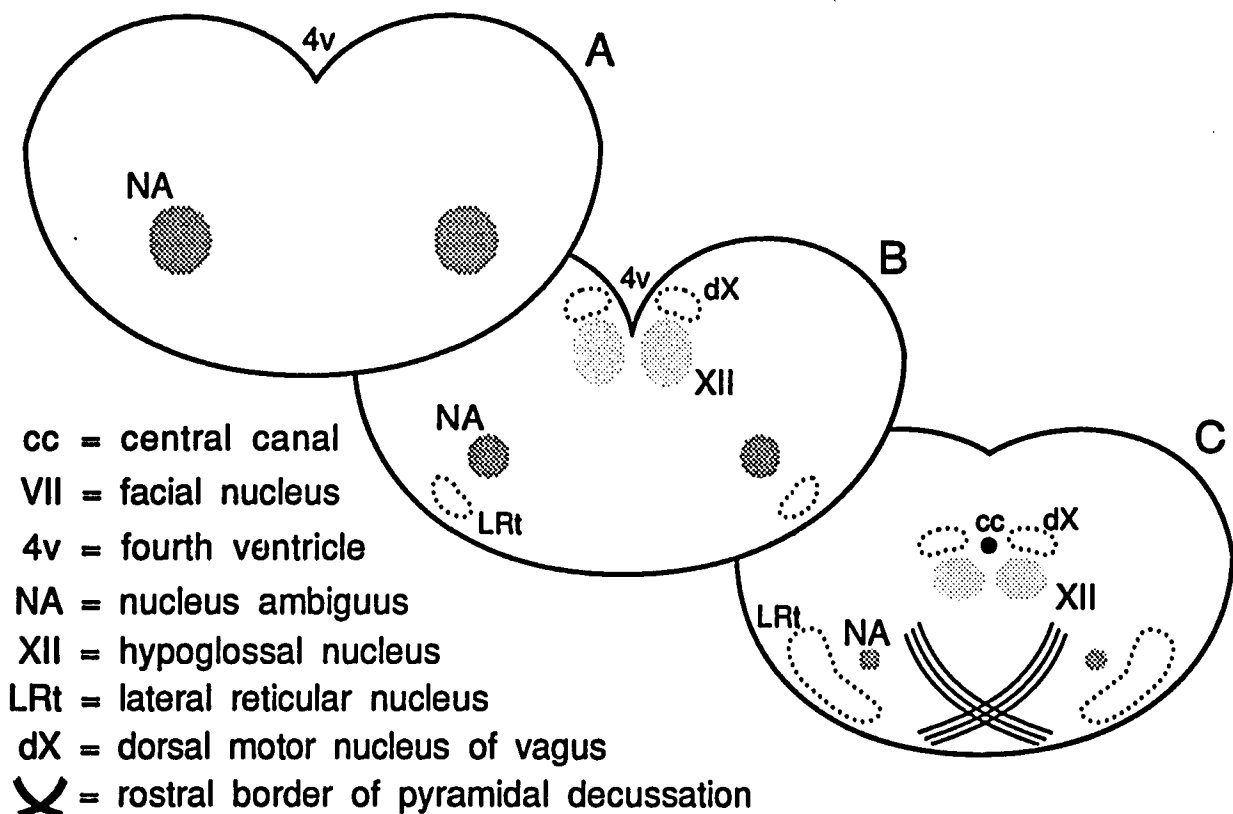
Figure 2.2

Brainstem Location of NA and XII

Lateral View of Lower Brainstem



Transverse Sections Through Brainstem



2.3.2 Types of Neurons in the Nucleus Ambiguus

NA has historically been considered to contain only large, special visceral efferent motoneurons which project to muscles in the URT (Furstenberg and Magielski, 1955; Bystrycka and Nail, 1985). Indeed, early studies by Bunzl-Federn in 1899 and Molhant between 1911 and 1913 demonstrated that NA contained neurons which projected to the larynx and pharynx (cited in Lawn, 1966a; 1966b). In 1955, Furstenberg and Magielski confirmed these early reports by using histological and electrophysiological methods to show definitively that nerve fibers projecting through the recurrent laryngeal nerve arose from NA, and that neurons in NA, provided efferent stimuli to muscles of the larynx. The neurons in NA were classified as motoneurons by Lawn who described the cells as "resembling the motor cells of the ventral horn of the spinal cord" (Lawn, 1966b: p309). In the early 1970's, the use of horseradish peroxidase (HRP) as a neural tract-tracer was developed (Kristensson and Olsson, 1971; LaVail and LaVail, 1972). Since that time, many studies have injected muscles of the larynx, pharynx, tongue (palatoglossus), and soft-palate (levator palati) with retrograde tracers and clearly showed prominent and specific labeling of motoneurons in NA (Gacek, 1975; Hinrichsen and Ryan, 1981; Schweizer et.al., 1981; Holstege et.al., 1983; Yoshida et.al., 1984; Van Loveren et.al., 1985; Hudson, 1986; Okubo et.al., 1987; Bieger and Hopkins, 1987; Grélot et.al., 1989a; Kitamura and Ogata, 1990; Patrickson et.al., 1991; Sokoloff and Deacon, 1992). That NA contains special visceral motoneurons has also been supported by recent electrophysiological studies (Delgado-García et.al., 1983; Yajima and Hayashi, 1983a, 1983b). In addition, studies using antibodies to choline acetyltransferase (ChAT), an enzyme characteristic of motoneurons, have shown strong immunoreactivity by the neurons in NA (Armstrong et.al., 1983; Mizukawa et.al., 1986; Tago et.al., 1989; Ichikawa and Hirata, 1990). This overwhelming evidence that NA contained motoneurons to branchiomic muscles of the URT has, until recently, precluded

considering the possibility that non-branchiomic motoneurons could be found in the same ventrolateral brainstem location.

In 1960, Bell studied the dorsal motor nucleus of the vagus (dX), the site of general visceral efferent motoneurons which project through the vagus nerve. In the course of this study, Bell found some degenerating neurons in NA after sectioning the vagus below the neck. Bell rejected the possibility that the chromalytic neurons in NA were general visceral motoneurons to the thoracic or abdominal viscera as it would have been counter to the understanding of topographic organization of the brainstem. However, over the ensuing years several physiological studies indicated that innervation of the viscera may not arise solely from dX and proposed the region of NA as an additional site of parasympathetic neurons (Kerr, 1969; Borison and Domjan, 1970; Nosaka et.al., 1978). Definitive results were not obtained until the application of HRP tract-tracing methodologies to the problem of brainstem neuroanatomy.

In 1980, Contreras and co-workers used HRP to trace the central origins of preganglionic parasympathetic fibers of several cranial nerves in the rat. They noted deviations from the generally accepted topographic organization of the brainstem. In particular, they found general visceral efferent neurons, not in the dorsal column, but in ventral regions of the brainstem. Additionally, an HRP study by Nosaka and co-workers (1979), also in the rat, demonstrated that some parasympathetic fibers in the cardiac branch of the vagus arose from the ventral brainstem as well. These studies indicated that Bell's findings of degeneration in NA may have been erroneously rejected based on incorrect assumptions of brainstem organization. Further studies demonstrated conclusively that NA contains general visceral efferent neurons in addition to the branchiomic motoneurons. For example, Coil and Norgren (1979) studied the central distribution of the entire subdiaphragmatic branch of the vagus nerve and found labeling in NA. Additionally, after injections of HRP into the trachea, lungs, heart, and stomach, individually, labelled neurons representing innervation to each organ were found in NA

(Kalia and Mesulam, 1980b). Similar studies have also identified parasympathetic neurons in NA which project to the pancreas (Weaver, 1980), the hepatic branch of the vagus (Rogers and Hermann, 1983), the stomach (Takayama et.al., 1982), the heart (Stuesse, 1982), and the carotid sinus (Housley et.al., 1987). Studies have been carried out primarily in the rat, although some reserachers have investigated the goat (Bell, 1960) and the cat (Kalia and Mesulam, 1980b). Although, little is known of the distribution of preganglionic parasympathetic neurons in primates these studies indicate that, at least in some mammals, NA contains branchiomic and parasympathetic motoneurons. Such an arrangement may play a significant role in coordinating upper and lower respiratory tract activity during respiration and even vocalization.

The above studies clearly demonstrate that NA plays a significant role in the control of the URT, as well as in the cardiac, respiratory, and digestive systems. As such, studies have focused on the physiology of the branchiomic and parasympathetic neurons and how it relates to peripheral control. In the course of these experiments, evidence has been presented for the presence of a third neuronal subtype in NA, the interneuron. Interneurons, or Renshaw neurons, provide inhibitory input to motoneurons and express the neurotransmitters glycine or γ -amino butyric acid (GABA) (Schwartz, 1985). Evidence for their presence in NA was first demonstrated in 1979 by DiMicco and co-workers. They noted that regulation of cardiovascular function might be related to the presence of GABA receptors. Thus, they injected a GABA receptor agonist into specific regions of the cat brainstem and monitored heart rate. DiMicco and co-workers found depression of heart rate only after injections into NA. Aside from providing physiological data supporting the presence of parasympathetic motoneurons in NA, it indicated that NA motoneurons were responsive to GABA. It was postulated that GABAergic input could arise from interneurons intrinsic to NA. Further evidence demonstrating interneurons in NA came from an electrophysiological study investigating the coordination of respiratory activity (Delgado-García et.al., 1983). Recordings from

cells in NA identified a non-motoneuronal population of cells that electrophysiologically appeared to be respiratory-related interneurons. The above studies provided indirect evidence for the presence of interneurons in NA. Further evidence was provided by Mugnaini and Oertel in 1985. Using an antibody against an enzyme specific for GABA synthesis, they demonstrated GABAergic neurons in the entire central nervous system of the rat. In the brainstem, Mugnaini and Oertel found that less than five percent of cells in NA were immuno-positive, although there was some immunoreactivity. Thus, the presence of interneurons in NA, although probably few in number, has been strongly suggested by studies employing histological, biochemical and electrophysiological methodologies.

2.3.3 Somatotopic Organization of Nucleus Ambiguus

Studies have sought to determine whether motoneurons in NA are somatotopically arranged, i.e., partitioned in discrete subdivisions of the nucleus with respect to the URT muscles innervated. Such partitioning has been shown to be functionally important by establishing patterns of peripheral neuromuscular control (Windhorst et.al., 1989). As this study focuses on developing motoneurons in NA, an understanding of the mature organization of the nucleus will enable the recognition of developmental periods during which such partitioning is established. This, in turn, will help define critical periods in the formation of URT neuromuscular control .

The organization of NA has been investigated in many different animals and the general morphology of the nucleus appears similar among mammals. As such, the first part of this discussion will focus on two well-characterized animals, the rabbit and the rat. This will define the basic subdivisions of the mammalian NA and establish a common terminology for understanding the locations of specific motoneuronal pools. The first comprehensive description of NA was by Lawn (1966a, 1966b) in the rabbit (figure 2.3). Reconstructions of NA from serial sections demonstrated what are referred

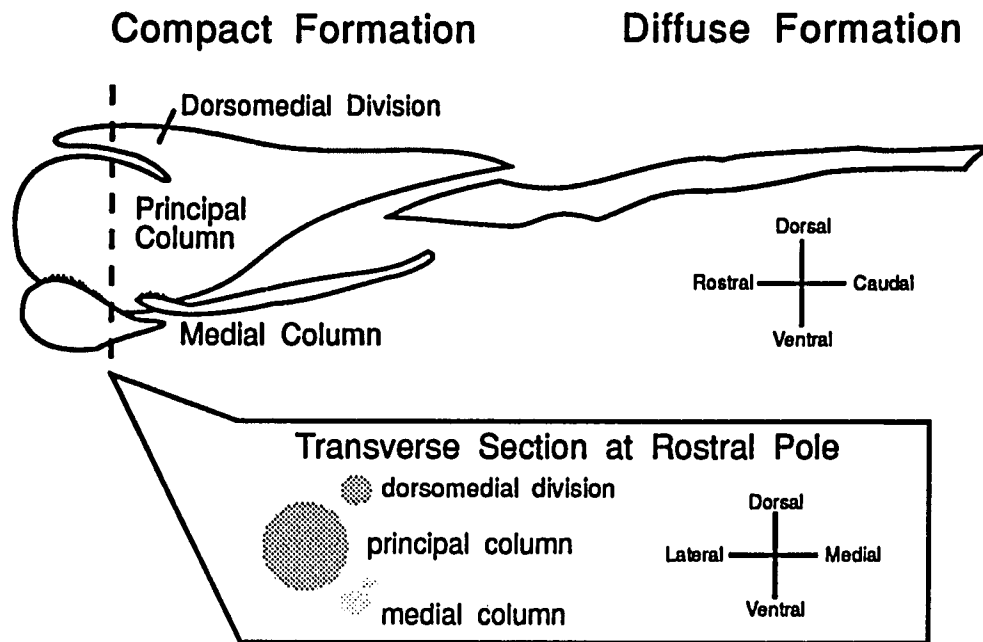
to as the compact and diffuse formations. The compact formation comprised only the rostral third of the nucleus yet contained 80% of the total neuronal population. This formation was further subdivided into three regions: 1) the principal column: the densest cluster of cells comprising the prominent round nucleus seen on cross-section; 2) the medial column: a scattered group of cells, although more compact rostrally, which lie medial to the principal column; 3) the dorsomedial division: a small group of cells closely associated with the rostral principal column. The diffuse formation comprised the rostral two-thirds of NA and was continuous with the compact formation. It contained a relatively small number of scattered neurons becoming increasingly separated along the rostral-caudal axis. Similar descriptions of the rabbit NA have been reported in recent studies using neural tracing methodologies (Ogata et.al., 1987; Okubo et.al., 1987; Kitamura et.al., 1991).

In addition to the rabbit, the morphology of the rat NA has also been well-characterized. Bieger and Hopkins (1987) defined a dorsal compact formation and a ventral external formation on a morphological and function basis (figure 2.3). The compact formation corresponded to the location of URT and esophageal motoneurons historically considered to comprise NA. This formation was subdivided into three regions: 1) the compact region: a dense clustering of cells comprising the rostral part of NA and corresponding to the principal and dorsomedial columns of the rabbit; 2) the semicompact region: medial to the compact region and similar in structure to the medial column of the rabbit; and 3) the loose region: found caudal to the compact and semicompact regions and analogous to the diffuse column of the rabbit. The external formation was ventrally located, less defined than the compact group, and consisted of parasympathetic neurons to the thoracic and abdominal viscera. This description of the rat NA has been corroborated by other researchers (Altschuler et.al., 1991; Núñez-Abades et.al., 1992). As noted, the descriptions of NA in the rabbit and rat have

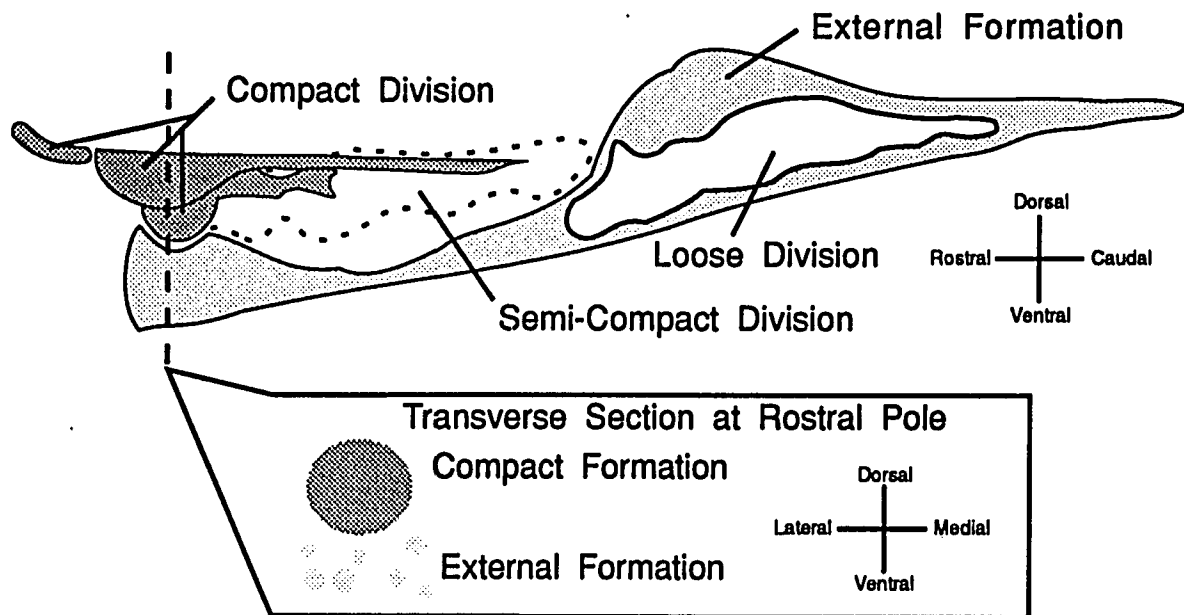
Figure 2.3

Anatomy of the Nucleus Ambiguus

Nucleus Ambiguus of the Rabbit (Lawn, 1966)



Nucleus Ambiguus of the Rat (Bieger and Hopkins, 1987)



established a general mammalian morphology and the following discussion of specific subsets of the motoneuron population will refer to this basic plan.

Experiments in the late nineteenth and early part of this century investigated the somatotopic organization of NA by histological and degenerative tract-tracing techniques (see review in Lawn, 1966a). These experiments provided a general understanding of the role of NA in URT innervation but left unresolved the specifics of the peripheral projections. As such, experiments employing similar, and refined electrophysiological techniques, reinvestigated the relationship between motoneurons in NA and their peripheral targets. For example, in 1955, Furstenberg and Magileski inserted an electrode into the monkey NA at several rostral-caudal levels and observed the effect of stimulation on the laryngeal muscles. In another study they sectioned the left recurrent laryngeal nerve and observed chromatolysis in NA. These studies demonstrated motoneurons to the cricothyroid muscle in the rostral-most NA, while motoneurons to adductor muscles were found most caudally. Degeneration studies by Lawn (1966a) in the rabbit showed similar results and provided information on pharyngeal and esophageal innervation. He demonstrated motoneurons to the pharynx, soft palate, and esophagus in the principal column (compact division); stylopharyngeus and cricothyroid motoneurons in the medial column (semicompact division); and the motoneurons of the recurrent laryngeal in the diffuse division (loose division). These degenerative and electrophysiological techniques provided a broad understanding of the somatotopic organization of NA. That is, they demonstrated the partitioning of neuronal innervation to groups of muscles (e.g., the location of laryngeal versus pharyngeal motoneurons), however, were limited in their ability to distinguish subpopulations of motoneurons projecting to individual URT muscles.

The localization of motoneurons of individual laryngeal and pharyngeal muscles became possible with the development of retrograde neural tract-tracing methods in the early 1970's (Kristensson and Olsson, 1971; LaVail and LaVail, 1972). Since that time,

numerous studies have investigated the central organization of motoneurons projecting to specific URT muscles. These studies have demonstrated a similar somatotopic organization in NA among a variety of mammals. For example, the location of pharyngeal motoneurons has been studied in the rat (Bieger and Hopkins, 1987; Núñez-Abades et.al., 1992), the cat (Holstege et.al., 1983; Van Loveren et.al., 1985; Grélot et.al., 1989a), the dog (Hudson, 1986), and the rabbit (Ogata, 1989; Kitamura and Ogata, 1990; Kitamura et.al., 1991). These motoneurons were consistently found in the rostral NA comprising parts of the compact and semicompact divisions. Stylopharyngeus motoneurons were in the rostral-most part of the compact division and parts of the semicompact division (Van Loveren et.al., 1985). In addition, the palatopharyngeus motoneurons were found throughout the compact formation extending from obex to the facial nucleus (Kitamura and Ogata, 1990). The superior pharyngeal constrictor motoneurons were in the compact and semicompact divisions slightly caudal to stylopharyngeus motoneurons (Bieger and Hopkins, 1987; Ogata, 1989). The middle and inferior constrictor (thyropharyngeus and cricopharyngeus) motoneurons were found to lie successively caudal and dorsal to those of the superior constrictor (Kitamura et.al., 1991). Esophageal motoneurons were found rostral to the majority of pharyngeal motoneurons and in a different dorsoventral plane of the compact division (Bieger and Hopkins, 1987; Kitamura et.al., 1988; Núñez-Abades et.al., 1992). Further studies in the extra-laryngeal URT found levator palati and palatoglossus motoneurons also in the rostral compact formation of NA (Holstege et.al., 1983; Sokoloff and Deacon, 1992). These studies demonstrated partitioning of rostral NA motoneurons with respect to embryologic and functional characteristics of the URT. For example, although NA motoneurons project to derivatives of the third, fourth, and sixth branchial arches, within the nucleus, glossopharyngeal motoneurons were located in the extreme rostral pole thus disitinct from neurons of other arches.

Aside from specific tracing studies of the pharyngeal muscles, HRP and other retrograde tracing techniques have allowed the localization of motoneurons in NA which project to the intrinsic muscles of the larynx. As in the pharyngeal region, laryngeal muscles appear to be partitioned along embryologic and functional lines. That is, tracing studies on the fourth and sixth arch nerves (i.e., the superior laryngeal and the recurrent laryngeal) indicated that although they both arise from NA, their motoneurons are in anatomically distinct location (Schweizer et.al., 1981). For example, studies indicated that motoneurons of the superior laryngeal nerve, as such to the cricothyroid muscle, were in the rostral-most part of NA in the medial or semicompact formation (Yoshida et.al., 1984; Patrickson et.al., 1991). In contrast, traces of the recurrent nerve indicated a large caudal group in the diffuse formation (Yoshida et.al., 1982; Hisa et.al., 1984). However, a smaller rostral group, overlapping neurons of the superior laryngeal nerve, has also been observed (Hinrichsen and Ryan, 1981; Patrickson et.al., 1991). Thus NA motoneurons of the fourth and sixth branchial arches are partitioned, at least in part, along embryonic boundaries.

The finding that motoneurons to the intrinsic muscles of the larynx were found in two different groups indicated that NA may be partitioned along other boundaries as well. Indeed, studies of the individual intrinsic laryngeal muscles have shown that this other boundary may, in fact, be function. As described above, rostral motoneurons of the cricothyroid muscle are overlapped by a group of recurrent laryngeal nerve motoneurons. Neural tracing studies have shown that this overlapping population of cells are a subset of motoneurons to the posterior cricoarytenoid muscle (Hinrichsen and Ryan, 1981). This group is distinct from the caudal group which contains, from rostral to caudal, more motoneurons to the posterior cricoarytenoid followed by motoneurons to the thyroarytenoid, lateral cricoarytenoid, and interarytenoid muscles (Pasaro et.al., 1981; Yoshida et.al., 1982; Pásaro et.al., 1983; Hisa et.al., 1984; Okubo et.al, 1987). The motoneurons to the posterior cricoarytenoid muscle in the rostral and caudal groups have

been shown to have different morphologies which may relate to the type of muscle fiber innervated (Pásaro et.al., 1983; Patrickson et.al., 1991). As such, this partitioning of laryngeal motoneurons may be related to the function of groups of muscles. For example, the cricothyroid and rostral posterior cricoarytenoid motoneurons may be in similar locations because they function together to open the glottic aperture (Megirian and Sherrey, 1991; Patrickson et.al., 1991).

This section has shown that motoneurons in NA are somatotopically arranged with respect to their embryologic origins and their projections to synergistic muscle groups. The various subdivisions and their motoneurons are shown in figure 2.4. Note that stylopharyngeal motoneurons are the most rostral, followed by motoneurons to fourth arch derivatives and finally motoneurons to intrinsic laryngeal muscles. Additionally, the rostral motoneurons project to muscles which may play a role in airway dilation, while the caudal group of motoneurons project to laryngeal adductors. This partitioning of NA along function grounds, may establish an anatomical substrate for the projection of afferent neurons to synergistic motoneurons. That is, higher neuronal centers initiating patterns of respiration and deglutition must project to specific groups of motoneurons in order to establish coordinated neuromuscular activity in the peripheral URT. The goal of this study is to define prenatal periods during which motoneuron partitioning and specific patterns of afferent innervation are established in NA. This, in turn, will elucidate critical periods in the formation of URT neuromuscular control.

2.3.4 Neuronal Input to the Nucleus Ambiguus

URT muscles innervated by NA, along with non-ambiguously innervated muscles, such as those of the tongue, must act in a coordinated manner to produce the functional neuromuscular patterns seen in respiration, deglutition, and vocalization. As such, motoneurons in NA receive afferent neuronal input from peripheral URT structures which serve to modulate and coordinate patterns of firing. Additionally, neurons within

Figure 2.4: Illustration of the somatotopic organization of the nucleus ambiguus (NA).

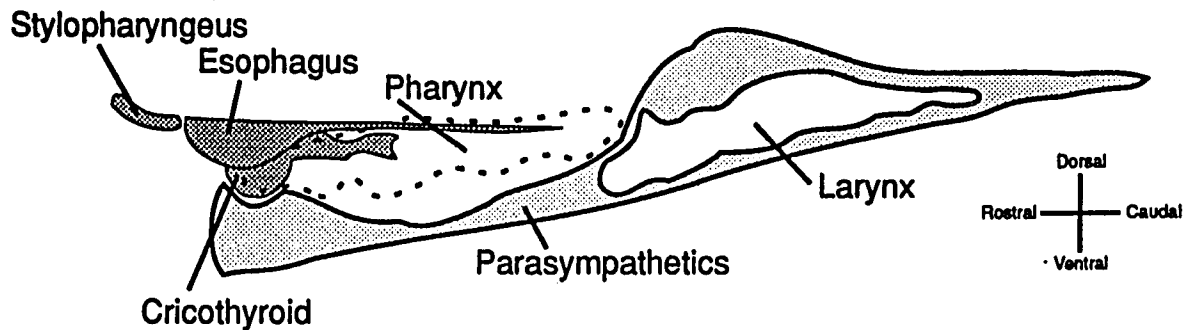
The top figure corresponds to the anatomy of the rat NA (refer to figure 2.3 and to text).

The lower diagram represents the anatomical location of the compact, semicompact and loose formations of NA and correlates the position of motoneurons to individual URT muscles with them (lines indicate extent of distribution). See the text for further discussion.

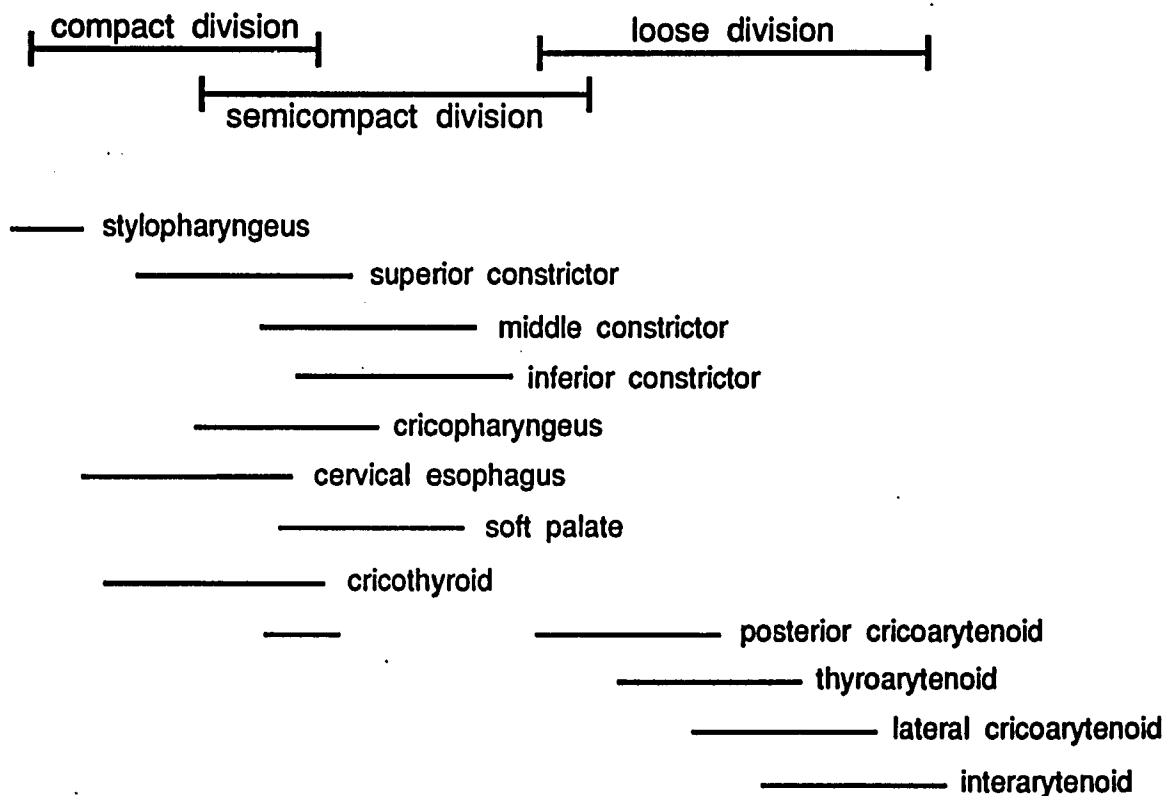
Figure 2.4

Somatotopic Organization of the Nucleus Ambiguus

Distribution of Motoneurons to URT Muscles
(Bieger and Hopkins, 1987)



Distribution of Motoneurons to Individual Muscles
(compiled from literature, see text)



the central nervous system project to NA motoneurons directing peripheral muscular patterns found during deglutition and vocalization. These afferent projections are largely established prenatally during periods in which they may influence the development of NA motoneurons. As such, an understanding of the neuronal centers which project to NA may help to characterize critical periods in the formation of the nucleus and the development of URT neuromuscular control.

2.3.4.1 Peripheral Input to the Nucleus Ambiguus

Of particular importance for survival of the organism are the neuromuscular responses to peripheral stimulation serving to protect and maintain the airway (see reviews, Sessle, 1975; Storey, 1975; Sasaki and Suzuki, 1976; Widdicombe and Tatar, 1988; Mathew and Sant'Ambrogio, 1988; Widdicombe et.al., 1988; Widdicombe, 1988). For example, stimulation of many regions of the URT can elicit reflex responses such as gags, coughs, swallows, or apneas (Johnson et.al., 1975; Storey and Johnson, 1975; Sweazey and Bradley, 1986). These reflexes imply the presence of an afferent neuronal system in the URT, capable of eliciting patterned and coordinated responses in the aforementioned brainstem motor nuclei. Thus, neuroanatomical studies have sought to identify the central projections of the afferent components of the glossopharyngeal and vagus nerves.

Tract-tracing studies of afferent fibers in the glossopharyngeal and vagus nerves have indicated projections to two brainstem regions: 1) the solitary tract; and 2) the spinal trigeminal nucleus. Projections to the solitary tract have been demonstrated in several animals including the lamb (Sweazey and Bradley, 1986), cat (Kalia and Mesulum, 1980a, 1980b; Lucier et.al., 1986) and the rat (Kalia and Sullivan, 1982; Housley et.al., 1987). The solitary tract has also been shown to receive afferent projections from the facial and trigeminal nerves (Contreras et.al., 1982). Thus, orofacial and aerodigestive afferents project to the same neuronal center allowing integration of sensory information from the entire URT. Furthermore, the nucleus of the solitary tract provides efferent

innervation to the trigeminal, facial, ambigular, and hypoglossal nuclei and may thus play a central role in coordinating URT reflexogenic responses (Travers and Norgren, 1983; Bystrycka and Nail, 1985).

Afferent fibers from the glossopharyngeal and vagus nerves have also been found to project to the spinal trigeminal nucleus about the level of obex (Sweazey and Bradley, 1986; Housley et.al., 1987). This region of the nucleus receives additional afferent input from the oral cavity via the trigeminal nerve (Wall and Taub, 1962; Sweazey and Bradley, 1986). Therefore, as observed in the solitary tract, afferent fibers from several regions of the URT appear to converge on a single neuronal center. In addition, like the solitary nucleus, the nucleus of the spinal trigeminal tract has been shown to project to URT motor nuclei (Borke et.al., 1983; Takada et.al., 1984). Thus, afferent fibers in the glossopharyngeal and vagus nerves project to two neuronal centers which also receive additional input from other regions of the URT. Furthermore, these centers have an efferent component which reciprocally projects to URT and orofacial motor nuclei. As such, peripheral stimuli may be integrated and processed in the sensory tracts and their respective nuclei. Subsequently, an efferent response would be transmitted to the brainstem motor nuclei resulting in specific neuromuscular patterns in the URT.

2.3.4.2 Central Input to the Nucleus Ambiguus

Motoneurons in NA, in addition to receiving afferent input from peripheral neurons and their sensory tracts and nuclei, receive input from central neurons of the midbrain, pons, and medulla. The sources of these higher neuronal inputs have been elucidated by tract-tracing studies in several species. Yoshida and co-workers (1991) injected HRP into the cat NA and found direct projections from the periaqueductal gray, the parabrachial nucleus, the nucleus of the solitary tract, the brainstem reticular formation, and the contralateral NA. Similar results were obtained in the rat by Travers and Norgren (1983) with additional projections found from the red nucleus, the superior

colliculus, the primary trigeminal sensory nucleus, the raphe nucleus, and the inferior olive. They did not attempt, however, to restrict the injection to within the boundaries of NA and some of the additional label may represent projections to neurons in the ventrolateral reticular formation.

In the above studies direct afferent projections from the forebrain to NA were not demonstrated, supporting other studies indicating that only primates contain direct corticobulbar projections (Kuypers, 1958a; Jürgens, 1976). With regard to NA, direct corticobulbar projections have not been seen in monkeys, however, have been demonstrated in humans (Kuypers, 1958b; Jürgens, 1976; Zeale, 1991). These direct connections may "override" subcortical projections to NA and account for our precise control of the larynx during vocalization (Zeale, 1991). The subcortical projections listed above, however, may serve a role in non-human phonatory behavior and help coordinate patterns of vocalization in all mammals. Furthermore, these regions may play critical roles in defining patterns of respiration and deglutition (see reviews, Sumi, 1975; Iscoe, 1988).

Some of the most complex patterns of neuromuscular activity in the URT are those associated with deglutition (Perlman, 1991). Motor patterns of deglutition have been found to be programmed and initiated in a region of the brainstem known as the swallowing center (Doty et.al., 1967; Lowe, 1981; Jean, 1984). This center has been localized to the medulla, in the reticular formation around the solitary tract, NA, and XII (Jean, 1984; Kessler and Jean, 1985). The swallowing center will be discussed further in the following section on XII and the tongue, but it should be noted that these regions of the brainstem have been shown to project to NA (Yoshida et.al., 1991). Thus, the swallowing center may serve to coordinate activity between muscles innervated by the trigeminal, facial, hypoglossal and ambigular nuclei.

Perhaps the most important function of NA innervated muscles is maintenance of the upper airway. This not only refers to the reflex protection of the airway described

previously, it includes accommodation of the laryngeal and pharyngeal musculature to changes in airflow during the respiratory cycle (Cohen, 1979; Widdicombe and Tatar, 1988; Bradford et.al., 1990). Indeed, many pharyngeal and laryngeal muscles exhibit respiratory activity during different phases of respiration (Merrill, 1970; Grélot et.al., 1989b; Woodson et.al., 1991; Amis et.al., 1992). Additionally, several areas of the medulla have been shown to be active during respiration, in particular, the dorsal respiratory group, in and around the solitary tract, and the ventral respiratory group, near NA (Amoroso et.al., 1954; Gromysz and Karczewski, 1976; Jiang and Shen, 1991; Richerson and Getting, 1992). In fact, several studies have suggested that some neurons of the ventral respiratory group are located within NA (Merrill, 1970; Ellenberger and Feldman, 1990a; Richerson and Getting, 1992). These respiratory centers, like the swallowing center, were found in regions of the brainstem shown to have direct and indirect connections to NA (Iscoe, 1988; Smith et.al., 1989; Ellenberger and Feldman, 1990b). Thus, URT neuromuscular activity during respiration could be determined in these centers by coordinated recruitment of specific motoneuronal pools in the orofacial and URT motor nuclei. For example, simultaneous contraction of the cricothyroid and posterior cricoarytenoid muscles has been shown to dilate the laryngeal aperture (Megirian and Sherrey, 1991). Phasic recruitment of the somatotopically arranged rostral NA motoneurons would activate these muscles concurrently, thus opening the airway during inspiration.

Although the most important function of the larynx is protection and maintenance of the airway, it is most closely associated with the production of sound. The sounds produced at the vocal folds can be modified by the pharynx and tongue, which in humans allows the production of articulate speech. Thus, as in respiration and deglutition, coordinated activity of URT motor nuclei is critical to the establishment of proper patterns of muscular activity in the peripheral URT during vocalization. As described at the beginning of this section, humans may have unique control of the URT during

vocalization because of direct cortical projections to motoneurons in NA. In contrast, cortical projections to NA in non-humans project polysynaptically primarily through regions of the pons and brainstem. These subcortical regions are also found in humans and may play a critical role in coordinating brainstem neuronal activity during vocalization. Among the regions thought to coordinate impulses between the cortex and NA are the periaqueductal gray and reticular formation (Schuller and Radtke-Schuller, 1988; Larson and Kistler, 1991) which have been shown to project to NA (Travers and Norgren, 1983; Yoshida et.al., 1991). Thus, NA receives input from higher neuronal centers which may establish peripheral patterns of vocalization and possibly integrate central respiratory drive with vocalizing behavior.

2.3.5 Summary

The nucleus ambiguus (NA) is located in the ventrolateral brainstem, caudal to the facial nucleus and dorsal to the lateral reticular nucleus. NA contains motoneurons which project through the glossopharyngeal and vagus nerves to muscles of the pharynx and larynx. On cross-section the nucleus has been subdivided into a compact and external formation. The compact formation and its subdivisions contain motoneurons of the larynx, pharynx, and esophagus. The ventrally located external formation primarily contains parasympathetic neurons to the thoracic and abdominal viscera. Rostrally, NA appears compact and clearly defined on cross-section. In the caudal regions NA is diffuse and less distinct.

Motoneurons in NA are somatotopically arranged with stylopharyngeus, cricothyroid and pharyngeal motoneurons found near the rostral pole. The caudal NA contains motoneurons to the intrinsic muscles of the larynx. Motoneurons of the posterior cricoarytenoid muscle are found in the rostral *and* caudal divisions. These motoneurons are of two distinct morphologies and may play different roles in the URT. For example, co-recruitment of the rostral cricothyroid and posterior cricoarytenoid motoneurons can

cause dilation of the laryngeal aperture, while recruitment of caudal NA motoneurons can cause vocal fold adduction.

The partitioning of motoneurons in NA allows afferent input to synapse on functional groups of motoneurons. Afferent input to NA arises from the periphery and from central neuronal projections. Peripheral inputs to NA are mediated through the solitary tract and spinal trigeminal tract. These sensory tracts receive afferent input from all the orofacial and URT cranial nerves and, in turn, project to their motor nuclei. Thus, peripheral stimulation can elicit a coordinated and appropriate response in the URT motor nuclei ensuring protection and maintenance of the upper airway. Central projections to NA are from swallowing, respiratory, and vocalatory centers. These regions project, not only to NA, but to the trigeminal, facial, and hypoglossal nuclei as well. As such, muscles in the URT act in a patterned and coordinated manner to function as a single system during respiration, deglutition and vocalization. The integration of central and peripheral afferents to NA, and the somatotopic arrangement of NA motoneurons, is largely established during prenatal development. However, little is known of the course of development in NA during this critical period.

2.4 Hypoglossal Nucleus

2.4.1 Introduction

The hypoglossal nucleus (XII) is found in the lower brainstem as a distinct, well-defined column of cells. It is the site of efferent innervation to muscles of the tongue as first demonstrated by Stilling (1843) in experiments on the rabbit and cat medulla. The hypoglossal nucleus (XII) is roughly centered about obex with its most rostral cells just caudal to the prepositus nucleus and its caudal border found at the pyramidal decussation (Taber, 1961). In transverse section, the nucleus lies along the brainstem midline near the fourth ventricle rostrally, and immediately ventral and ventrolateral to the central

spinal canal caudally (figure 2.2) (Lewis et.al., 1971). The left and right nuclei closely approximate one another throughout their extent and communicating dendritic fibers have been observed crossing the midline between them (Cooper, 1981a; Wan et.al., 1982). Further characterization of XII has focused on several neuroanatomical criteria: 1) the morphological and functional classification of hypoglossal neurons; 2) the somatotopic organization of XII with respect to its projections to muscles of the tongue; and 3) the anatomy and functional implications of afferent neuronal projections to XII.

2.4.2 Classification of Hypoglossal Neurons

The first study to classify neurons in XII was done by Ramón y Cajal in 1909 (cited in Cooper, 1981a). Using the Golgi method of staining neurons he described two morphologically distinct classes of motoneurons. These were called "internal" or "external" motoneurons based upon the nature of their dendritic projections (Travers, 1985). That is, internal neurons had dendrites whose projections remained within the boundary of XII, while external neurons had dendrites which projected outside XII to surrounding neuronal centers. Further morphological classification of hypoglossal neurons was not pursued until relatively recently. In 1976, Odutola used the Golgi technique and described three different morphologies for hypoglossal neurons: fusiform, round-to-globular, and triangular-to-multipolar. All these cells were characterized as Golgi type I cells which means they projected out of XII, presumably to muscles of the tongue. As such, Odutola, like Ramón y Cajal, identified only primary motoneurons in XII. Several other studies also examined hypoglossal neurons, and although findings varied with regard to morphological descriptions, all these studies similarly concluded that XII contained only motoneurons (Hashimoto and Palay, 1965; Alley, 1973; Cooper, 1981a).

While morphological investigations of XII demonstrated only motoneurons, electrophysiological studies of the nucleus have been inconclusive. Some studies showed

synaptic potential latencies in and around XII indicative of interneuron modulation while others failed to repeat these results (Sumi, 1969; Lodge et.al., 1973; Sumino and Nakamura, 1974; Lowe, 1981). Prompted by these contrasting reports, researchers re-examined the neurons of XII histologically in attempts to demonstrate the presence or absence of a non-motoneuronal population of neurons. Cooper, who early in 1981 indicated only motoneurons in Golgi stained sections of XII, reported later in the year the presence of smaller neurons with morphologically distinct characteristics (Cooper, 1981b). A subsequent study in the rat further characterized these smaller neurons. Boone and Aldes (1984) injected the rat tongue with HRP in order to label motoneurons in XII. They then examined XII electron microscopically and indeed observed a large number of type I motoneurons containing HRP reaction product. They also found several smaller neurons with ultrastructural features characteristic of interneurons. These cells were not labeled with HRP implying a neuronal function other than innervation of tongue muscles.

Recent investigations of the function of cells in XII have employed the use of immunocytochemical methods to demonstrate neurotransmitters or the enzymes necessary for their biosynthesis. Initial studies looked for acetylcholine, the excitatory neurotransmitter found at neuromuscular junctions. The demonstration of its biosynthetic enzyme, choline acetyltransferase (ChAT), in neurons of the spinal cord and brainstem, largely, defines motor nuclei and their respective motoneurons. Immunocytochemical studies of ChAT expression have indeed shown heavy labeling in the majority of neurons of XII (Armstrong et.al., 1983; Connaughton et.al., 1986; Tatehata et.al., 1987; Jones and Beaudet, 1987).

Interneurons also contain a characteristic neurotransmitter, γ -aminobutyric acid (GABA) and its associated biosynthetic enzyme, glutamic acid decarboxylase (GAD). In 1985, Mugnaini and Oertel demonstrated a small number of neurons in XII that were immuno-positive for GAD. As this study was an overview of the entire rat CNS, they

provided few details as to the specifics of expression in XII. An immuno-cytochemical study in the following year, although not investigating GABA expression, demonstrated that the small neurons in XII did not express ChAT and thus were not motoneurons (Connaughton et.al., 1986). A subsequent study, specific for XII and for GABA, demonstrated its expression in the small neurons of XII and not in the large motoneurons (Takasu et.al., 1987). Additionally, these small neurons ultra-structurally resembled interneurons, however, synaptic contact between motoneurons and the GABA-positive neurons was not noted. Takasu and Hashimoto (1988) furthered this study by demonstrating histologically that the axon of a small proposed interneuron did indeed synapse on the dendrites of a hypoglossal motoneuron. These studies have shown that there are neurons other than motoneurons in XII. Furthermore, ultrastructural and immunocytochemical data indicates that these small neurons are likely to be interneurons and thus may play a role in modulation of neuromuscular control of the tongue.

2.4.3 Somatotopic Organization of the Hypoglossal Nucleus

In attempts to understand better the control of the tongue, XII has been divided into subnuclei based upon histological appearance in order to define functionally distinct subunits. The number of observed subnuclei varies between species which may be reflective of the comparative complexity of tongue structure and function (Barnard, 1940). For example, Ulinski (1973) observed no histologically distinct subnuclei in XII of the snake (*Constrictor constrictor*), while the cat (*Felis catus*) had two subnuclei (Uemura et.al., 1979); the monkey (*Macaca fascicularis*) five (Sokoloff and Deacon, 1992); and Mysticete whales (*Balaenoptera physalus* and *B. muscularus*) four (Jansen and Korneliussen, 1977). Division of XII into histologically defined subnuclei, however, may be, in part, reflective of subjective observer bias as evidenced by differences in classifications within species. For example, the rat XII has been described as having as few as three subnuclei (O'Reilly and Fitzgerald, 1990) and as many as nine (Odutola,

1976). These differences may also be due to changes in nucleus appearance along the rostral to caudal axis. That is, some subnuclei do not extend the entire rostral-caudal length of XII and different numbers of subnuclei are visible at different brainstem levels (Odutola, 1976; Chibuzo and Cummings, 1982). Furthermore, while some researchers recognize dorsal and ventral subdivisions, others have proposed medial and lateral divisions (Barnard, 1940; Odutola, 1976; Uemura-Sumi et.al., 1988; Sokoloff and Deacon, 1992). These different descriptions of XII have made it difficult to demonstrate histologically a functional organization among hypoglossal motoneurons.

In order to determine whether XII is somatotopically arranged with respect to peripheral muscular innervation, experimental, rather than histological, studies employing degeneration and neural tracing techniques were employed. These studies helped identify specific regions of XII from which individual tongue muscles, and thus patterns of tongue activity, were controlled. Although the researchers indicated soma position in XII by citing their own nomenclature for various subdivisions, the results will be presented here with regard to anatomical location in the nucleus so that these studies may be directly compared.

In 1971, Lewis and co-workers showed conclusively that functional patterns of the tongue were topographically represented in the peripheral and central hypoglossal nervous system of the rat. Peripheral partitioning of function was shown by stimulating the lateral and medial branches of the hypoglossal nerve. When the lateral branch was stimulated the tongue generally retracted and when the medial branch was stimulated it caused the tongue to protrude. Central partitioning of neuromuscular activity was then demonstrated by differential loss of staining for cholinesterase in XII after sectioning of peripheral branches of the nerve. In this manner, when the lateral branch which contained axons to retrusor muscles was cut, loss of staining was seen in the dorsal part of XII. Similarly, when the medial branch, and thus the protrusor neurons, were cut, staining loss was observed in the ventral part of XII. This work established a general

pattern of hypoglossal nucleus organization: 1) that the dorsal part of XII contains motoneurons which innervate the retrusor muscles and are distributed in the lateral branch of the hypoglossal nerve; and 2) that the ventral part of XII contains motoneurons which innervate the protrusor muscles and are distributed in the medial branch of the hypoglossal nerve.

By the late 1970's neural tract tracing techniques employing substances such as HRP were widely used and were able to identify neurons projecting to a single muscle. Using HRP, Watkins (1978) localized motoneurons innervating the geniohyoid to the ventrolateral region of XII in the rat. Additionally, Watkins showed that motoneurons in the dorsal region of XII projected to the hyoglossus and styloglossus muscles. He was unable, however, to distinguish innervation patterns of the genioglossus or of the intrinsic tongue muscles. Krammer and co-workers (1979) obtained the same results for hyo- and styloglossus as well as geniohyoid, and were able to localize genioglossus innervation to the ventromedial part of XII. Furthermore, using HRP they confirmed the findings of Lewis and co-workers (1971) that the lateral branch arose from dorsal XII motoneurons and the medial branch from ventral neurons.

The observed pattern of neuromuscular partitioning seen in the rat appears to be well conserved among mammals. For example, in the cat, the lateral and medial branches of the hypoglossal nerve contain axons of motoneurons in the dorsal and ventral subdivisions of XII, respectively (Uemura et.al., 1979). Furthermore, genioglossus innervation arises ventromedially, geniohyoid ventrally, and hyo- and styloglossus dorsolaterally. This general pattern has also been observed in the dog (Chibuzo and Cummings, 1982). Although tracing of individual intrinsic tongue muscles was not performed in these animals, injections into the body of the tongue indicating innervation arising from the more medial regions of XII (Uemura et.al., 1979; Chibuzo and Cummings, 1982; Satomi et.al., 1985). Interestingly, injections of the body of the tongue in both the cat and dog have also resulted in the labeling of some motoneurons in the

facial motor nucleus (Chibuzo and Cummings, 1982; Satomi et.al., 1985). This may indicate that remnants of the second branchial arch may persist in the body of the tongue in the mature organism (Chibuzo and Cummings, 1982; Sadler, 1985).

In order to understand patterns of neuromuscular innervation in the human tongue, neural tract tracing experiments have been performed in monkeys. Using a variety of macaques (*Macaca fuscata*, *M. nemestrina*, *M. speciosa*, and *M. irus*), Uemura-Sumi and co-workers (1981) found similar patterns of innervation as in non-primate mammals. The subdivisions of the primate XII, however, are arranged obliquely relative to the ventrodorsal axis and although the partitioning seen in other mammals is conserved, the nomenclature is slightly different. For example, the lateral branch of the hypoglossal nerve arises from the dorsal division of XII in non-primates but from the lateral and laterodorsal divisions in primates (Uemura-Sumi et.al., 1981). Regardless of differences in nomenclature, in all mammals geniohyoid motoneurons have been found most ventrally, genioglossus motoneurons medially, and hyoglossus and styloglossus motoneurons dorsolaterally (Uemura-Sumi et.al., 1981; Sokoloff and Deacon, 1992).

Although prior studies in the dog and cat indicated intrinsic innervation of the tongue arising from the medial divisions of XII, those studies were not specific for the individual muscles. The larger size of the primate tongue, however, allowed the injection of HRP into individual intrinsic tongue muscles. An extensive study by Sokoloff and Deacon (1992) on 27 macaques (*M. fascicularis*) elucidated the patterns of central partitioning of intrinsic muscle innervation and indicated that the earlier studies were not entirely accurate. They showed that transversus and verticalis are indeed innervated by the medial pool of hypoglossal motoneurons but that superior and inferior longitudinalis are innervated by the lateral division of XII. Furthermore, Sokoloff and Deacon showed that injections of the palatoglossus outside the body of the tongue resulted in labeling of the nucleus ambiguus and no label in XII. This may be indicative of the embryologic origins of the muscle. Additionally, within several muscles, location in XII along the

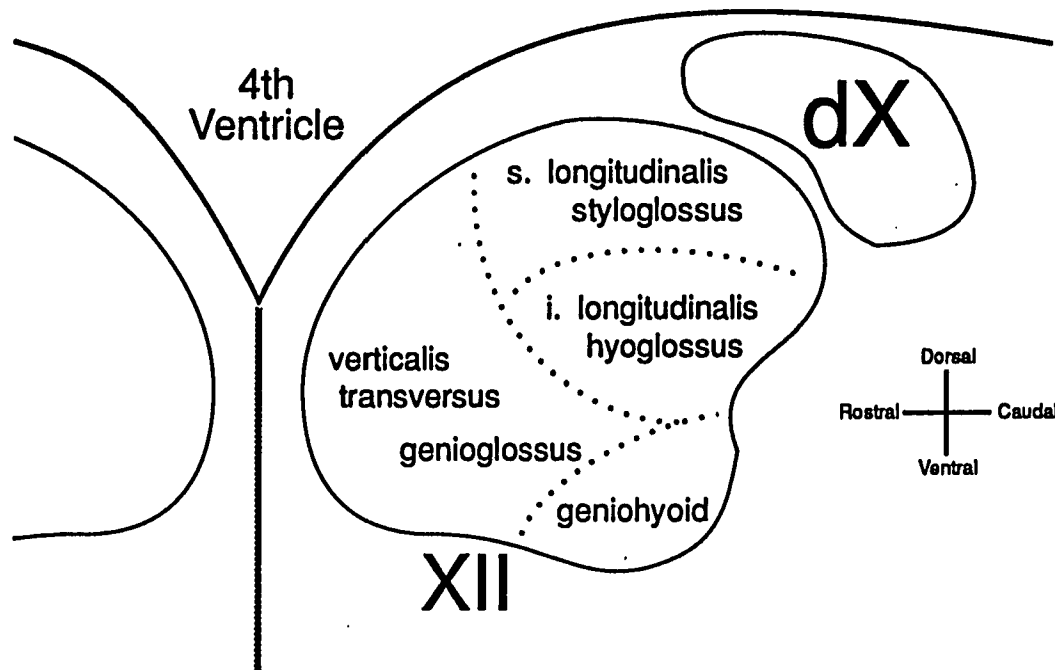
rostrocaudal axis was correlated with proximity of the injection to the anterior tip of the tongue. This indicates somatotopic organization of XII along all three orthogonal axes.

The studies on the somatotopic organization of XII are summarized in figure 2.5. This figure clearly demonstrates the partitioning of motoneurons in XII with respect to function of the innervated tongue muscle. That is, retrusor muscles are innervated by dorsolateral motoneurons and protrusor muscles are innervated by the ventromedial motoneurons. In addition, geniohyoid which neither inserts nor originates in the body of the tongue, yet is important in tongue movement, is innervated by an extreme ventral division of XII. These results also support the work of Abd-El-Malek who in the 1930's argued that function is not partitioned by whether the muscle is intrinsic or extrinsic to the tongue but by coordinated recruitment of muscles from both groups. Indeed, these results show that motoneurons to intrinsic and extrinsic muscles often overlap in XII as long as their stimulation results in the same action. For example, motoneurons to the styloglossus (extrinsic) and superior longitudinalis (intrinsic) muscles are grouped together dorsolaterally because they act together to produce tongue retrusion.

2.4.4 Neuronal Input to the Hypoglossal Nucleus

Functional organization of XII allows higher cortical and brainstem neuronal centers to elicit specific peripheral responses by synapsing on related groups of neurons. For example, movement of the tongue could be elicited by afferent neuronal input to the ventromedial XII. This would recruit motoneurons projecting to muscles which, when activated in a coordinated neuromuscular sequence, cause protrusion of the tongue. The actions of the tongue, however, in establishing critical neuromuscular patterns for respiration, deglutition and vocalization, are more complex than simple protrusion and retrusion. As such, afferent neuronal input to motoneurons of XII arises from many regions of the central and peripheral nervous systems. Afferent input to XII can be

Figure 2.5
Somatotopic Organization of the Hypoglossal Nucleus



Tongue Muscles		Location in XII (nomenclature as per Sokoloff and Deacon, 1992)
Intrinsic Muscles	Extrinsic Muscles	
superior longitudinalis	styloglossus	Lateral Subdivision (retrusor) lateral nucleus pars dorsalis
inferior longitudinalis	hyoglossus	lateral nucleus pars ventralis
.....		
transversus; verticalis		Medial Subdivision (protrusor) medial nucleus pars medialis
	genioglossus	medial nucleus pars intermedialis
.....		
	geniohyoid	Ventral Subdivision

divided into two broad categories: 1) input from the periphery ; and 2) input from central neuronal populations.

2.4.4.1 Peripheral Input to the Hypoglossal Nucleus

Whether hypoglossal motoneurons receive afferent input from the periphery has historically been controversial (Weddell et.al., 1940; Blom, 1960; Lowe, 1981; Travers, 1985). This is, in large part, due to the lack of sensory ganglia associated with the nerve (Blom, 1960; Zapata and Torrealba, 1971). Thus, studies have sought to demonstrate afferent receptors in the tongue and sensory fibers in the hypoglossal nerve. These studies have been both anatomic and electrophysiologic in nature and have established that a proprioceptive afferent system does indeed relay information from the tongue and other orofacial muscles to the motoneurons of XII.

Proprioception is the sense of position and movement in peripheral structures usually conveyed by muscle spindles in the body of the muscle and mechanoreceptors in the joint capsule (Martin, 1985). The tongue, however, does not move a fixed joint and proprioception must therefore rely on muscle spindles or similar structures in the muscles of the tongue. In 1953, Cooper demonstrated spindles in the intrinsic and extrinsic muscles of the human tongue. She showed a predilection for these receptors in the middle of the tongue with few seen near the tip or base. Additionally, most receptors were found in the superior longitudinalis with very few in the inferior longitudinalis. Cooper also demonstrated spindles in the tongue of the monkey, but found no spindles in the cat or the lamb. However, in the cat, afferent discharges in the hypoglossal nerve could be elicited electrophysiologically by stretching the tongue thus indicating the presence of some form of receptor (Cooper, 1954). A similar study in the cat by Blom (1960) also found no morphologically distinct muscle spindles in the tongue but did demonstrate the presence of receptors electrophysiologically.

Further studies on proprioceptors in the tongue helped to establish the concept that the primate tongue had morphologically distinct muscle spindles while lower mammals were lacking in such structures (Bowman, 1968; Bowman and Combs, 1968). However, re-examination of the rat tongue and associated muscles began to challenge this hypothesis as muscle spindles were found in the geniohyoid (Maier, 1979). Further investigation confirmed the presence of spindles in the geniohyoid and also demonstrated such receptors in the intrinsic inferior longitudinalis muscle of the rat tongue (Smith, 1989). This finding was supported by O'Reilly and Fitzgerald (1990) who demonstrated the presence of spindles in the hyoglossus and genioglossus muscles as well. Although, spindles were demonstrated in the rat tongue, they were found only in muscles arranged longitudinally and are thus restricted to providing information for movement along a single axis. This is in contrast to findings in primates where receptors have been found in all muscle planes enabling response to activity in three-dimensions, and possibly indicating underlying differences in the function of the tongue (Bowman, 1968; Smith, 1989). For example, in rats, the tongue is most active during deglutition and the movement is primarily in the anteroposterior direction. However, in primates, complex neuromuscular patterns associated with vocalization may require monitoring of tongue movement and position in all directions.

The recent histological demonstrations of mechanoreceptors in the non-primate tongue, have actually trailed electrophysiological studies which have, for some time, shown the presence of afferent fibers in the hypoglossal nerve. In fact, as described above, even though Cooper (1954) and Blom (1960) failed to demonstrate, histologically, muscle spindles in the cat tongue, their electrophysiological data showed afferent fibers in the hypoglossal nerve. Further electrophysiological studies have also demonstrated proprioceptive afferents in the hypoglossal nerve of several species (Bowman and Combs, 1968; Sumi 1969; Zapata and Torrealba, 1971; Tanaka, 1975; Nakamura et.al., 1978; Takata et.al., 1979; Lowe, 1981; O'Reilly and Fitzgerald, 1990).

These studies, however, showed afferents fibers in only the distal-most regions of the hypoglossal nerve. While this implies the presence of proprioceptors in the tongue that leave within the hypoglossal nerve, it does not provide information as to how and where these afferents enter the brainstem. The proximal course of these sensory fibers is not intuitively evident because XII contains no sensory neurons and the hypoglossal nerve has no associated ganglia. As such, studies have sought to trace the path of these afferents back to the brainstem anatomically and electro-physiologically.

Experiments in monkeys showed that central responses to peripheral hypoglossal nerve stimulation could be obliterated by sectioning the dorsal roots of spinal nerves C2 or C3 (Bowman and Combs, 1968). This indicates that afferents may leave the tongue through the hypoglossal nerve and follow the ansa cervicalis to the dorsal root ganglia in the upper cervical region. This pathway has also been observed in the cat (Zapata and Torrealba, 1971), the dog (Chibuzo and Cummings, 1981), and the rat (O'Reilly and Fitzgerald, 1990). A second route by which hypoglossal afferents may return to the brainstem has often been observed in conjunction with the ansa cervicalis pathway. This pathway supposes a communicating branch between the hypoglossal and vagus nerves and indicates that the sensory neurons may reside in the nodose ganglion (Zimny et al., 1970; Zapata and Torrealba, 1971; Tanaka, 1975; O'Reilly and Fitzgerald, 1990). Indeed, in some studies, small communicating branches between the vagus and hypoglossal nerves have been observed (Zapata and Torrealba, 1971; O'Reilly and Fitzgerald, 1990). The use of horseradish peroxidase, however, indicates that the pathway between hypoglossal and vagus nerves may be more complex than by a single communicating branch. Chibuzo and Cummings (1981) postulated a neural pathway from the hypoglossal nerve to the lingual nerve to the chorda tympani to the facial nerve to the auricular branch of the vagus nerve to the vagal ganglia. Regardless of the pathway, these studies have clearly provided an anatomical and physiological basis by

which discharge of hypoglossal motoneurons may be modulated by afferents responding to movement of the tongue.

As noted in chapter 1, the tongue is an integral part of a complex muscular system. Its activity must therefore be coordinated with other muscles to establish patterns of respiration, deglutition, and vocalization. As such, studies have investigated whether hypoglossal motoneurons may respond, not only to proprioceptive afferents from the tongue, but to afferents from other URT muscles and structures. Such experiments have typically stimulated nerves other than the hypoglossal and recorded electrical changes in motoneurons of XII. These studies have shown that activity of hypoglossal motoneurons may be modulated by feedback from branches of three nerves: 1) the trigeminal; 2) the glossopharyngeal; and 3) the vagus.

The lingual nerve, a branch of the trigeminal, may carry proprioceptive signals from mechanoreceptors in superficial and deep portions of the tongue (Porter, 1966). As such, several studies have used electrophysiological techniques to investigate the presence of a lingual-hypoglossal reflex (Sauerland and Thiele, 1970; Sauerland and Mizuno, 1970; Morimoto et.al., 1972; Schmitt et.al., 1973). These studies have demonstrated conclusively that such a reflex exists, however, the effect on hypoglossal activity may be either facilitatory or inhibitory (see review Lowe, 1981). In general, it appears that lingual nerve stimulation is inhibitory on both protrusive (P) and retrusive (R) motoneurons but can also produce initial excitatory potentials in R motoneurons (Morimoto et.al., 1972; Lowe, 1984). Other branches of the trigeminal nerve may also elicit reflex responses from hypoglossal motoneurons. For example, stimulation of auriculotemporal nerve branches to the temporomandibular joint can cause facilitatory responses in P motoneurons (Lowe, 1984). Additionally, stimulation of muscles of mastication (e.g., masseter, temporalis, anterior digastric) have been shown to be inhibitory on all hypoglossal motoneurons and to reduce tongue reflexes evoked by other nerves (Sauerland and Thiele, 1970; Sauerland and Mizuno, 1970; Morimoto et.al., 1972;

Schmitt et.al., 1973). Reciprocally, stimulation of hypoglossal afferents are inhibitory on masseter motoneurons (Nakamura et.al., 1978). Thus, these reflexes may serve to protect the anterior tongue during mastication (Sauerland and Mizuno, 1970).

Whereas the lingual nerve supplies general sensation to the anterior two-thirds of the tongue, the glossopharyngeal nerve supplies the posterior third. In addition, the glossopharyngeal nerve supplies sensation from the walls of the pharynx. Stimulation of the glossopharyngeal nerve, as in the lingual nerve, evoked mixed responses in XII (Blom, 1960; Duggan et.al., 1973; Lowe, 1981). That is, glossopharyngeal stimulation caused either facilitation or inhibition of P and R motoneurons. It has not yet been determined whether the differential effects on hypoglossal motoneurons may reflect stimulation of the lingual or pharyngeal branches of the glossopharyngeal nerve and thus indicate underlying functional significance (Lowe, 1984). The glossopharyngeal reflex was also shown consistently to inhibit the lingual-hypoglossal reflex while itself being inhibited by activity of muscles of mastication (Sauerland and Thiele, 1970; Hunter and Porter, 1974). Thus, the glossopharyngeal reflexes may act, like the lingual-hypoglossal reflex, in protection of the tongue during mastication.

Stimulation of the superior laryngeal nerve (SLN) has been shown to initiate the complex neuromuscular patterns of swallowing (Doty et.al., 1967; Kessler and Jean, 1985). As the tongue plays a significant role during deglutition, studies investigated the effect of SLN stimulation on hypoglossal motoneuron activity. It was found that stimulation of the SLN evoked a facilitatory potential on P motoneurons and caused protrusion of the anterior tongue (Sumi, 1969; Schmitt et.al., 1973; Miller and Bowman, 1974). This demonstrates the presence of a reflex protrusion of the tongue after laryngeal mucosal stimulation which could function during normal swallowing and also be important in airway protection (Schmitt et.al, 1973).

In summary, the above studies have demonstrated that motoneurons in XII receive a great deal of afferent input from peripheral structures. In addition to the tongue,

reflexive signals arise from the muscles of mastication, the oral mucosa, and the oro- and laryngo-pharynx. These reflexes can be excitatory and inhibitory and may be specific for protrusive or retrusive motoneurons. The way in which these reflexes are integrated to coordinate patterns of deglutition and respiration is not yet clear. It should be considered that reflexes between the muscles of mastication and the lingual and glossopharyngeal nerves are necessarily inhibitory to protect the tongue and to allow programmed patterns of tongue activity to emerge during mastication. Furthermore, facilitation of protrusive activity after SLN stimulation may protect the airway by eliciting a swallow to remove an obstruction or by opening the pharynx for forceful expulsion such as during a cough. These are a few examples of the reflexes critical for proper URT control and, as such, the maintenance of the organism. As such, the connections between the afferent neurons, motoneurons of XII and peripheral muscles must be specific so as to elicit the proper response. These connections are largely established in the prenatal organism, however, little is known of the developmental events ensuring such synaptic specificity.

2.4.4.2 Central Input to the Hypoglossal Nucleus

The above studies have clearly demonstrated the presence of a peripheral proprioceptive system between orofacial muscles and motoneurons of XII. However, motoneurons in XII have no recurrent collaterals and the number of neurons in XII with Renshaw-like properties appears to be relatively low (Sumi, 1969; Lodge et.al., 1973; Mugnaini and Oertel, 1985). Furthermore, electrophysiological studies have demonstrated latencies indicative of polysynaptic connections between the afferent fibers and hypoglossal motoneurons (Porter, 1966; Porter, 1967; Miller and Bowman, 1974; Takata et.al., 1979). These findings, in conjunction with the many pathways by which proprioceptive afferents return to the central nervous system, indicate that neuronal centers outside XII may play a role in the regulation of discharge patterns of hypoglossal

motoneurons. Indeed, several regions have been postulated to contain the interneurons in these feedback loops.

As afferent fibers from the tongue and distal hypoglossal nerve have been shown to enter the brainstem through cervical spinal roots and vagal rootlets, studies have sought to demonstrate projections from the central sensory nuclei of these nerves to XII. Proprioceptive fibers from cervical spinal roots ascend through the dorsal column-medial lemniscal system and afferent information to XII may thus be relayed through neurons of the reticular formation (Martin, 1985). Indeed, injection of HRP into XII in the rat and cat has shown input from neurons in many portions of the medial and lateral reticular formations (Borke et.al., 1983; Travers and Norgren, 1983; Takada et.al., 1984). The projection of hypoglossal afferents through the vagus nerve indicates a course ultimately directed at the solitary tract and nucleus. Tract-tracing studies have shown some direct projections from the solitary nucleus to XII (Borke et.al., 1983; Takada et.al., 1984), however, evidence indicates that most connections are polysynaptic, mediated by neurons in the reticular formation (Schmitt et.al., 1973; Travers and Norgren, 1983; Takada et.al., 1984). Thus, proprioceptive afferents from the tongue seem to project to XII via polysynaptic pathways ultimately mediated by interneurons in the reticular formation.

As described in the previous section, motoneurons in XII also receive afferent input from sensory fibers in specific branches of the trigeminal, glossopharyngeal and vagus nerves. The sensory component of the glossopharyngeal and vagus nerves is the solitary tract and nucleus which has been shown to project mono- and polysynaptically to motoneurons in XII. The trigeminal nerve, however, projects to different sensory nuclei, specifically the semilunar ganglia, the principal trigeminal sensory nucleus, the mesencephalic nucleus, and the spinal trigeminal nucleus (Kelly, 1985). Thus, additional pathways for the various trigeminal-hypoglossal reflexes needed to be demonstrated.

Degeneration studies showed that proprioceptive afferent neurons from the muscles of mastication return to the mesencephalic nucleus and project directly to XII through

Probst's tract and indirectly via the descending juxtatrigeminal fascicle (Mizuno and Sauerland, 1970). The Probst tract projections to XII, however, may terminate just outside the nucleus, possibly synapsing on motoneurons with externally projecting dendrites (Borke et.al., 1983; Takada et.al., 1984). These results provide a route for the inhibition of hypoglossal motoneurons during mastication. Investigation of the lingual-hypoglossal reflex utilized injections of HRP into the tongue and showed no labeling of neurons in the mesencephalic nucleus (Chibuzo and Cummings, 1981). Rather, labeled neurons were found in the trigeminal ganglia. No direct pathways between the trigeminal ganglia and XII have been found and it is postulated that a polysynaptic pathway is involved (Lowe, 1981). Additional projections to XII have been demonstrated in the dorsal part of the spinal trigeminal nucleus (Borke et.al., 1983; Takada et.al., 1984). This region of the nucleus has been shown to receive innervation from regions of the oral cavity (Wall and Taub, 1962).

The above studies have demonstrated the large number of brainstem neuronal centers which project to motoneurons of XII. These mediate the various orofacial reflexes thus integrating and coordinating actions of the tongue with neuromuscular activity in the rest of the URT. One of the most complex functions, requiring the coordination of many muscles and nerves and critical to the survival of all mammals, is deglutition. In addition to the aforementioned pathways coordinating activity reflexively, a specific region of the brainstem has been shown to initiate deglutition and is known as the swallowing center (Doty et.al., 1967; Lowe, 1981; Jean, 1984). This center has been localized to the medulla, in the reticular formation around the solitary tract, NA, and XII (Jean, 1984; Kessler and Jean, 1985). Studies of firing patterns of neurons in the swallowing center indicate that it serves to program the muscular activity of deglutition and not simply serve as a relay for peripheral reflexes (Jean, 1984; Kessler and Jean, 1985). The interneurons of the swallowing center can be activated by central stimulation or by peripheral stimulation of the SLN (Kessler and Jean, 1985). Both pathways result in the

same patterns of firing in XII which consists of coordinated facilitatory and inhibitory signals serving, in part, to activate P motoneurons (Lowe, 1981). It should be noted that other regions of the brainstem have been proposed as additional swallowing centers based on anatomical and physiological data. This includes a region of the caudal pontine tegmentum which projects to XII, as well as NA and the trigeminal motor nucleus (Holstege et.al., 1983).

In addition to the swallowing center, the reticular formation around NA and XII also contains the respiratory centers. This region, and its functions, were previously discussed in section 2.3.4.2. However, it should be noted that motoneurons in XII have been shown to fire in phase with the expiratory phase of respiration (Sumi, 1969). In addition, P motoneurons are active during the phase of inspiration (Lowe, 1984). This indicates the presence of underlying connections between medullary centers of respiration and motoneurons of XII.

Aside from brainstem swallowing and respiratory centers, afferent fibers have been shown to project to motoneurons of XII from other central neuronal pools. For example, tract-tracing studies have shown projections to XII of serotonin containing neurons in the medial tegmental field (Holstege and Kuypers, 1977; Holstege et.al., 1977; Manaker et.al., 1992) and norepinephrine containing neurons of the nucleus subceruleus (Aldes, 1990; Aldes et.al., 1992). Of particular interest, however, are projections from cortical regions as these may be important in control of the tongue during vocalization. Studies in the cat have found no direct cortico-hypoglossal projections but, rather, have found projections to the brainstem reticular formation (Walberg, 1957; Kuypers, 1958a; Travers and Norgren, 1983). Additionally, the rat has also been shown to lack direct corticobulbar projections (Valverde, 1962; Borke et.al., 1983). In contrast, studies have found direct monosynaptic connections, along with indirect connections, between the cortex and brainstem motor nuclei in primates, including humans (Kuypers, 1958b; Bowman and Combs, 1969a, 1969b; Jürgens, 1976; Sokoloff, 1990). These direct

projections may add an additional level of control to XII, and thus the tongue. It may be postulated that by bypassing the reticular formation, and thus swallowing, respiratory and reflex interneurons, specific and novel patterns of neuromuscular activity may be evoked in the tongue important for vocalization.

2.4.5 Summary

The hypoglossal nucleus lies in the dorsomedial brainstem and supplies efferent innervation to the muscles of the tongue. The motoneurons in XII are somatotopically arranged so as to represent the functions of intrinsic and extrinsic tongue muscles. Retractor motoneurons are found in the dorsolateral nucleus and protruder neurons in the ventromedial region. Motoneurons to geniohyoid are located in the extreme ventral part of XII, often enmeshed with hypoglossal axons projecting toward the periphery. A small number of non-motoneurons have been identified in XII. They make up less than five percent of the population and morphologically and biochemically appear to be interneurons.

Proprioceptive afferents from the tongue are conveyed in the distal hypoglossal nerve but enter the brainstem through the vagus nerve and upper cervical spinal roots. Additional mechanoreceptor afferent fibers from the tongue travel through the lingual nerve and establish a lingual-hypoglossal reflex. Other reflexes are also observed between orofacial nerves and the hypoglossal. These include a masseteric-hypoglossal reflex, a glossopharyngeal-hypoglossal reflex, and a superior laryngeal nerve-hypoglossal reflex. These afferents project to XII polysynaptically by way of trigeminal sensory nuclei, the solitary tract and nucleus and the reticular formation. Additional input to XII comes from reticular formation swallowing and respiratory centers and other neuronal centers in the pons and midbrain. Direct cortico-hypoglossal projections are found only in primates and may play a role in establishing neuromuscular patterns of vocalization.

Hypoglossal motoneurons are a "link" between brainstem and cortical neurons and peripheral muscles of the tongue. The afferent and efferent connections to and from hypoglossal motoneurons are highly specific and critical for the coordinated activity of the tongue during respiration, deglutition, and vocalization. These specific patterns of innervation are largely established during prenatal development, however, little is known of the course of prenatal development in XII.

Chapter 3

Motoneuron Cell Death

3.1 Introduction

3.1.1 Cell Death During Development

Shortly after the union of oocyte and spermatozoon, the newly formed zygote undergoes the first of many cell divisions needed to create a complete organism. New cells are produced with each successive mitosis and subsequently differentiate to begin to define organs and organ systems. This process of cell formation, i.e., cell birth, dominates our perception of *normal* prenatal development and the contribution of cell death to prenatal maturation is often overlooked. In fact, it is only relatively recently that the occurrence of developmental cell death began to be widely accepted and investigated by the scientific community (Saunders, 1966)

Cell death during normal development is a naturally occurring process and should not be confused with pathologic occurrences of cell death. Naturally occurring cell death takes place in *specific tissues at specific developmental stages*. It serves to remove cells no longer needed and to define morphologies necessary for future organ and organ system function (Hurle, 1988; Michaelson, 1991). This chapter focuses on a specific occurrence of natural cell death essential to the formation of motoneuron pools in the nervous system: motoneuron cell death (MCD). MCD is but one example of naturally occurring cell death, a process categorized into four groups: morphogenetic, metamorphic, phylogenetic, and histogenetic (Glucksmann, 1951).

Morphogenetic Cell Death The shape of an organism and many of its organs are largely determined by regional differences in the rates of cell proliferation. However, certain morphologies cannot be attained by differential cell birth but rather are formed by specific occurrences of cell death. For example, developing limbs initially appear as flat paddles. Morphogenetic cell death removes parallel rows of cells in the distal portions of these structures, defining digits from the remaining tissues (Saunders and Fallon, 1966). This pattern of digit formation by cell death is seen in all members of the Amniota (e.g.,

mammals, birds and reptiles) (Hinchliffe, 1981). However, in amphibians digit formation occurs by cell proliferation and not cell death (Hinchliffe and Johnson, 1980). This indicates that patterns and occurrences of cell death have arisen under evolutionary selection and may reflect common lineage as well as key evolutionary events necessary for the emergence of more complex morphologies and organ systems.

Metamorphic Cell Death Metamorphic cell death occurs in organisms which pass through distinct developmental stages. For example, insects pass through pupal, larval and adult stages and frogs pass through a tadpole stage. The transition to the adult organism often requires the removal of structures unique to the immature stage. For instance, the transition from tadpole to adult frog requires the elimination of the tadpole tail to achieve the adult morphology. These structures are removed by a process of naturally occurring cell death. Although, this may be considered an example of morphogenetic cell death, its occurrence within the context of passing from one distinct life cycle to another, has been categorized as metamorphic cell death (Glucksmann, 1951; Lockshin, 1981; Kimura and Truman, 1990).

Phylogenetic Cell Death Organisms which do not undergo metamorphosis, e.g., mammals, may still develop structures embryologically that do not persist into later developmental stages. However, some of these structures may be found to persist in phylogenetically older organisms. For example, lower mammals such as the rat have an artery in the middle ear called the stapedia artery. Adult humans normally have no such blood vessel. However, during human embryogenesis, a stapedia artery forms in the middle ear, passes through the stapes bone, and is subsequently removed by a process of naturally occurring cell death (Davies, 1973). The space left by the degenerated stapedia artery is evident in postnatal humans, found between the crus of the stirrup-like stapes. There is a small percentage of individuals who have not undergone this particular process of phylogenetic cell death and indeed retain a congenital stapedia artery. The removal of structures that may be considered phylogenetic remnants is an example of

morphogenetic cell death specifically referred to as phylogenetic cell death (Glucksmann, 1951).

Histogenetic Cell Death Histogenetic cell death is associated with the differentiation of organs and tissues (Hinchliffe, 1981). It occurs in many developing systems, for example, the formation of male and female genitourinary tracts by selective degeneration of the Mullerian and Wolffian ducts, respectively (Glucksmann, 1951; Maraude et.al., 1982). In recent years, histogenetic cell death has been shown to occur most prominently in the developing nervous system where over 50% of cells in a given population may be eliminated (Oppenheim, 1991). This chapter is devoted to the occurrence of histogenetic cell death in developing motor pools: motoneuron cell death.

The above categories provide teleological descriptions of cell death and, as such, may be used to describe a single occurrence of cell death in several ways (e.g., morphogenetic and phylogenetic). That these occurrences of cell death can not be categorized in more distinct terms implies an underlying similarity among these developmental phenomena. In fact, all the above categories are examples of naturally occurring cell death and, as such, exhibit similar morphological and biochemical changes during the degenerative process. The following section demonstrates these similarities and contrasts these natural processes with the more familiar pathological causes of cell death.

3.1.2 Natural versus Pathological Cell Death

As the occurrence of natural processes of cell death began to be accepted, questions arose as to the possible differences between such normal cell death and the death of cells under pathological conditions (Saunders, 1966). Early investigations focused on the appearance of dying cells and proposed two classifications of cell death based on observed morphological changes during the degenerative process: apoptosis and necrosis (Kerr et.al., 1972; Wyllie et.al., 1980). In general, natural or developmental cell death proceeds by the process of apoptosis whereas pathologic cell death occurs by necrosis

(Hurle, 1988; Walker et.al., 1988). This paradigm is not absolute and some morphological aspects of necrosis have been observed developmentally (Chu-Wang and Oppenheim, 1978a; Hinchliffe, 1981). In fact, recent research has suggested reclassifying the types of cell death based on these observations (Clarke, 1990; Oppenheim, 1991). Nonetheless, within the current classification, the differences in the cellular and molecular processes underlying the morphological changes in apoptosis and necrosis reinforce the concept of naturally occurring cell death as a normal part of development (Lockshin and Zakeri, 1991; Oppenheim et.al., 1992).

3.1.2.1 Necrosis

Deleterious changes in the extracellular environment may be sufficiently severe as to result in the death of cells in that region (Trump and Ginn, 1969; Kerr and Harmon, 1991). The process by which these cells die and the characteristic degradative changes in cell structure is called necrosis. Necrotic changes in cell structure help to distinguish pathologic cell death from the naturally occurring cell death found during normal development (see reviews Wyllie et.al., 1980; Walker et.al., 1988).

The first necrotic changes in cell structure as observed by electron microscopy are reversible and an elimination of the extracellular insult can prevent the death of these cells (Jennings et.al., 1975). Necrotic changes are first seen primarily in the cytoplasm with mild edema, dilation of the endoplasmic reticulum, dispersal of ribosomes, and swelling of the mitochondrial matrix (Trump and Ginn, 1969; Beaulaton and Lockshin, 1982). Continued environmental insult to the cell results in dramatic swelling of the mitochondria and formation of granular densities within the mitochondrial matrix (Kerr, 1969; Kerr, 1970). These mitochondrial observations are thought to represent changes in cell morphology and physiology from which the cell can not recover, that is, the removal of the insult at this time will not prevent cellular necrosis (Jennings and Reimer, 1981; Wyllie, et.al., 1980). Subsequent changes include the rupture of organelle and

cytoplasmic membranes, as well as the dissolution of ribosomes and lysosomes (Searle et.al., 1982).

Necrotic changes in the nucleus are less dramatic with early disturbances evident as loose clumping of chromatin around the periphery of the nucleus. This is seen by light microscopy as uniform chromatin clumping known as pyknosis followed by focal margination called karyorrhexis (Walker, et.al., 1988). As the necrotic process continues there is progressive dispersal of chromatin clusters and finally rupture of the nuclear membrane (Searle et.al., 1982). In the final stages of necrosis, cell boundaries become indistinct and a contiguous sheet of cell ghosts are seen rimmed by inflammatory cells in the neighboring viable tissue (Wyllie et.al., 1980; Wyllie, 1981).

The mechanism behind these morphological changes is thought to involve changes in cell permeability and the inability to regulate cell volume (Trump and Ginn, 1969). Insult to the cell can directly injure the plasma membrane or effect membrane proteins involved in ion-pumping activity (Duvall and Wyllie, 1986). This allows movement of cations into the cell which changes the osmotic balance and causes cellular edema. The increase in intracellular calcium activates phospholipases which accelerate membrane degradation and contribute to changes in cell organelle morphology and function (Jennings and Reimer, 1981). For example, mitochondrial damage leads to a loss of oxidative metabolism and probably accounts for the irreversibility of the necrotic process after matrix damage. Additionally, damage to lysosomes releases hydrolytic enzymes which degrade proteins and DNA during the late stages of necrosis (Beaulaton and Lockshin, 1982). The resultant DNA fragments are of random size, indicative of the lack of cellular control over degradation during necrosis (Afanas'ev et.al., 1986).

Although the mechanism of cell death following pathological insult is conserved, the insult itself can be of many forms. Necrosis can result from cell injury due to hypoxia, ischemia, hyperthermia, complement-mediated membrane disruption, membrane active toxins, metabolic inhibitors, autolysis *in vitro*, and as a result of tumor growth (Walker,

et.al., 1988). It is important to note that necrosis is caused by harsh changes in the cell's environment and not by any intracellular factors.

3.1.2.2 Apoptosis

Naturally occurring cell death, as seen in developing organisms, generally occurs by a different process: apoptosis (Kerr et.al., 1972). The most significant feature of apoptosis is that the degenerative process arises from within the cell. That is, in contrast to necrosis, apoptosis can be described as "cell suicide" (Williams, 1991).

As with necrosis, there is a well conserved sequence of changes in cellular morphology observable during apoptosis (see reviews Wyllie et.al., 1980; Wyllie, 1981; Walker et.al., 1988; Kerr and Harmon, 1991). However, unlike necrosis, the first degradative changes are primarily seen in the nucleus. At the start of apoptosis the nucleolus disperses and the chromatin condenses into large granular masses along an often abnormally convoluted nuclear membrane (Wyllie et.al., 1981; Kerr and Harmon, 1991). Early extranuclear changes include mild cytoplasmic condensation and loss of adhesions between the apoptotic cell and neighboring viable cells (Kerr, 1971; Searle et.al., 1982). In later stages of apoptosis, increased cytoplasmic condensation results in crowding of organelles and the formation of clear cytoplasmic vacuoles (Wyllie et.al., 1980). Finally, characteristic protuberances called "apoptotic bodies" form on the cell surface and frequently bud-off into the intercellular space (Kerr, et.al, 1972). These bodies contain intact organelles and sometimes nuclear fragments. They are phagocytosed by tissue macrophages or sometimes by other non-apoptotic cells in the tissue (Wyllie, 1981). Under the light microscope these apoptotic changes are difficult to discern. This is due to the often small size of apoptotic bodies and the rapid dissolution and removal of the dying cells (Searle, 1982; Walker et.al., 1988; Kerr and Harmon, 1991). In apoptosis, the cytoplasmic changes are under cellular control unlike the

passive edematous changes seen in necrosis (Clouston and Kerr, 1979; Lockshin and Beaulaton, 1981).

The mechanism of apoptosis is difficult to study because apoptotic cells are often surrounded by many healthy cells. This is in contrast to necrosis which affects large numbers of contiguous cells (Bellairs, 1961; Searle et.al., 1982). It is known, however, that unlike necrosis, apoptosis is an energy dependent process, i.e., ATP levels remain normal whereas in necrosis loss of ATP to membrane pumps is thought to be a precipitating factor (Wyllie, 1981). Additionally, apoptosis is genetically controlled as protein synthesis have been shown to be necessary for initiation and progression of the apoptotic process (Wyllie et.al., 1984; Lockshin and Zakeri, 1991). Furthermore, chromatin condensation is due to specific endogenous endonucleases which cleave double stranded DNA between nucleosome linker regions. This is contrary to the findings of random DNA fragmentation in necrosis (Duke et.al., 1983; Afanas'ev, 1986).

Apoptosis most commonly occurs in the elimination of cells during fetal and embryonic development (O'Connor and Wytenbach, 1974; Beaulaton and Lockshin, 1982). Additionally, apoptosis serves to regulate organ size and cell turnover in the adult. It is also a characteristic feature in the immune system being closely associated with T-lymphocyte function (Wyllie et.al., 1980). There are some extracellular factors that can induce apoptosis. These include ionizing radiation, mild hyperthermia, and certain cancer-chemotherapeutic drugs (Searle et.al., 1982). Apoptotic cells are also seen in some tumors along with necrosis (Kerr and Harmon, 1991).

The distinctions between necrosis and apoptosis are helpful in understanding the normal occurrence of cell death during embryonic and fetal development. Although morphologically developmental cell death may exhibit changes characteristic of both processes, the underlying mechanisms resemble those found only in apoptosis. Indeed, it has recently been suggested that the term "apoptosis" should no longer describe morphological changes, but rather should refer to the biochemical and molecular

mechanisms common to all *naturally* degenerating cells (Lockshin and Zakeri, 1991). These include the active participation in the degenerative processes by the cell's own genome and the induction of these "suicide" genes by specific extracellular signals. The remainder of this chapter will focus on a particular occurrence of natural cell death in the developing central nervous system, motoneuron cell death.

3.1.3 Definition of Motoneuron Cell Death (MCD)

During the development of the central nervous system, cells are produced at the ependymal layer lining the ventricles and central canal. Those cells originating from the basal plate in the brainstem and spinal cord become motoneurons. The number of motoneurons produced prenatally exceeds the number found in the postnatal organism. During prenatal development these excess motoneurons are removed by a process of histogenetic cell death (see reviews Grinnell and Herrera, 1981; Oppenheim, 1991). This period, and process, has been referred to by many terms including: naturally occurring cell death, cell death of motoneurons, normal motoneuron death, and motoneuron cell death. We have adopted the term motoneuron cell death, abbreviated MCD.

MCD, unlike many other examples of developmental cell death, is not a genetically dictated phenomenon. That is, the number of cells destined to die, and the identities of those cells, is not predetermined by the genome. Rather, the course of MCD is influenced by epigenetic signals from surrounding neurons, target muscles, and even the extra-uterine environment. Thus, MCD represents a critical period in the development of neuromuscular control. Furthermore, demonstration of its natural course in specific motor nuclei will elucidate those gestational periods in which the establishment of neuromuscular specificity may be susceptible to pathological insult. This, in turn, may help to explain postnatal pathologies associated with inefficient, uncoordinated muscular activity (Oppenheim, 1991). Specifically, understanding the course of MCD in URT motor nuclei may provide a template upon which to predicate the myriad of URT

"incoordination" pathologies seen in newborn humans (Friedland et.al., 1991, 1992, 1993). The remainder of this chapter will focus on the discovery and characterization of MCD. Additionally, this chapter will review the nature and effects of epigenetic signals on the course of MCD and the role these play in the establishment of neuromuscular control.

3.2 Historical Background

At the turn of this century, neuroembryologists were debating the validity of two competing theories on the origins of peripheral nerve fibers (Harrison, 1906). The reticular theory, supported by Hensen and Golgi, held that axons developed from the condensation of many protoplasmic bridges between Schwann cells called plasmodesms. These plasmodesms were postulated to connect a continuous network of nerve cells with their peripheral targets. In contrast, the neuron theory, put forth, in large part, by His and Ramón y Cajal, postulated that the axon was the projection of a single contiguous cell to its target (see reviews by Hamburger, 1980; 1988). In order to resolve this conflict, Ross Granville Harrison initiated what are considered the first experimental neuro-embryological studies (Hamburger, 1988). These studies, in addition to addressing the above question, began to characterize the neuromuscular system and the developmental interactions between its component parts.

In 1906, Harrison reported a series of experiments on the frog embryo spinal cord. In one experiment he removed a portion of the cord, in another, he transplanted it to a foreign location, and in a third experiment he removed the target. Harrison's results showed that nerve fibers arose *from* the spinal cord, regardless of location or the presence of a target. Thus, he demonstrated, in support of the neuron theory, that the axon is an extension of the neuron that developmentally grows out towards the periphery. As such, Harrison showed that neuromuscular control requires the interaction of two separate cell

populations: neurons and muscle fibers. This result formed the basis of additional studies by Harrison into the nature of the interaction between developing neurons and their target muscles.

Harrison (1904), questioned whether developing muscle was dependent upon innervating neurons for the initiation of differentiation. He thus removed the spinal cord of frog embryos and assessed muscle formation. He found that myoblast differentiation and myofiber organization proceeded as normal indicating a lack of neuronal influence. Three years later, Harrison (1907) addressed the opposite question: Is neural development dependent upon the innervated muscle? He transplanted a frog limb bud to the flank and found innervation, but by the flank portion of the spinal cord. Furthermore, the branching pattern within the limb was normal, i.e., thoracic neurons were induced to distribute like lumbar neurons. Thus, Harrison showed that some aspects of neural development were indeed directed by the target tissue.

The relationship between neurons and their target tissue was further investigated in 1909 by Shorey. Shorey removed the limb buds from developing chick embryos and found pronounced hypoplasia of the innervating sensory and motor neuron pools. It was concluded that the target tissue determined the number of neurons by influencing the differentiation of neuroblasts. Around 1920, Detwiler, a student of Harrison's, performed similar experiments but transplanted the removed limb to a heterotopic site (Detwiler, 1919; Detwiler, 1920). He similarly found that the dorsal root ganglia deprived of the target limb had fewer cells than normal. In addition, he found that the ganglia innervating the heterotopic limb were hyperplastic as compared to normals. Thus a relationship was again shown to exist between the number of developing neurons and the nature of the peripheral target. However, unlike Shorey, Detwiler found no differences in the motoneuron pools deprived of or overloaded with target tissue.

In 1932, Viktor Hamburger joined the lab of Frank Lillie and was asked to repeat experiments done over 20 years prior by Shorey, one of Lillie's students (Hamburger,

1980, 1988). The goal was to reconcile the discrepancy between Shorey and Detwiler with regard to the effect of limb bud extirpation on developing motoneuron pools (Hamburger, 1992). Using refined techniques of limb bud removal, Hamburger (1934) was able to show that the amount of hypoplasia in the motor column and in the spinal ganglia was proportional to the amount of muscle and skin removed, respectively. In further experiments, Hamburger (1939) showed that adding tissue, i.e., a supernumerary limb, created a hyperplasia in the corresponding motor column and dorsal root ganglia. Both Hamburger and Detwiler proposed theories by which the innervated tissue influenced cell number in the respective neuronal pool. They proposed that pioneer nerve fibers would send signals back to the central nervous system and indicate the size of the innervation field. This would, in turn, recruit the proper number of cells from an undifferentiated pool to complete the motor column or sensory ganglion. As Hamburger wrote, however, "Both explanations had the advantage that they could explain hypo- and hyperplasia by the same mechanism. A disadvantage was that they were wrong." (Hamburger, 1988:p3540)

In 1942, Rita Levi-Montalcini and Giuseppe Levi repeated Hamburger's experiments and arrived at a different mechanism. They studied the number of cells present in spinal ganglia at various developmental stages and found that the hypoplasia is the result, not of reduced proliferation, but of cell degeneration. Shortly after this, Hamburger and Levi-Montalcini (1949) began to collaborate and showed that cell degeneration was indeed the correct mechanism. This study focused on spinal ganglia and, as such, the role of cell degeneration in the motor horn was neglected for many years. Indeed, after finding that sensory neurons without a target die, Hamburger and Levi-Montalcini's research turned toward finding a possible trophic factor that would promote the survival of these cells. This search led to the Nobel Prize winning discovery of nerve growth factor (NGF) in the early 1950's (Levi-Montalcini and Hamburger, 1951, 1953).

In 1958, Hamburger returned to examine motor hypoplasia after limb bud removal in the chick embryo. Similar studies were carried out through the 1960's most notably by Cowan and Wenger (1967), Hughes (1961), and Prestige (1967). These studies began to show that motoneuron degeneration was not limited to the site of target depletion but that a natural process of cell degeneration was also occurring on the control side. This led to the gradual acceptance of motoneuron cell death in the nervous system as a normal part of development. In the early 1970's the idea was raised that naturally occurring cell death and cell death after limb bud removal may be similar phenomena (Cowan, 1973). Additionally, it was postulated that the addition of limb buds actually rescued cells that would otherwise naturally die (Hollyday and Hamburger, 1976; Oppenheim et.al., 1978). This led to an explosion of investigation into the causes and factors influencing naturally occurring motoneuron cell death. Led largely by Ronald Oppenheim, research in the field has moved from anatomical descriptions of cell death to the role of muscles and afferents in cell death, to current focuses on trophic factors and the cellular and molecular mechanisms underlying their actions.

3.3 Epigenetic Influences on MCD

The discovery of naturally occurring motoneuron cell death grew out of experiments designed to explore the dependence of developing neural centers on their peripheral targets (Hamburger, 1958; Hughes, 1961; Cowan and Wenger, 1967). As such, it was noted that the fate of developing motoneurons was, at least in part, determined by their peripheral target (Hughes, 1961; Hughes, 1965; Cowan, 1973; Hollyday and Hamburger, 1976). Over the past 15 years many studies have examined the characteristics of the innervated muscles which influence motoneuron survival. Additionally, the role of afferent neurons in providing developmental cues to immature motoneurons has recently been investigated. The findings of these studies will be addressed in the following

subsections and provide a background for understanding how MCD is a perturbable, environmentally sensitive process representing a critical period in the development of neuromuscular control.

3.3.1 Muscle Size

The dependence of the developing nervous system on target tissue was, as described above, discovered early in this century. These results showed a relationship between target size and the post-embryonic number of differentiated neurons. However, at the time, the results were interpreted as alterations of neurogenesis and not as modifications of a natural degenerative phenomena. Once the natural occurrence of motoneuron death was recognized efforts were directed at defining the specific parameters of muscle size that related to motoneuron survival. These studies established the basic tenets that: 1) if target muscle size is increased, motoneuron cell death is decreased, and 2) if target muscle size is reduced, motoneuron cell death is increased.

3.3.1.1 Increased Target Muscle Size / Decreased MCD

In their seminal paper describing for the first time a naturally occurring degenerative process in spinal ganglia, Hamburger and Montalcini (1949) put forth the idea that adding to the periphery may prevent central neuronal degeneration. At the time, the increase in motor columns following peripheral overloading (Hamburger, 1939) was not addressed. By the early 1970's the occurrence of a natural degenerative process of motoneurons was gaining acceptance and the issue of hyperplasia following limb bud transplantation was again raised. Cowan (1973) suggested that additional muscle prevents the degeneration of motoneurons that would normally die. In order to address this specific problem Hamburger (1975) first charted the normal course of MCD in the lateral motor column of the chick embryo spinal cord. As Harrison did in 1904, this work by Hamburger helped (re)establish the chick embryo spinal cord as the model for

the majority of studies into the mechanisms of neuromuscular development. After the normal course of MCD was known, detailed analysis of the effect of a transplanted limb bud could be carried out.

In 1976, Hollyday and Hamburger removed the right limb bud of a donor chick embryo and transplanted it just rostral to the right limb bud of an acceptor embryo. Thus, the right lumbar motor column would have twice the muscle to innervate. Embryos were sacrificed before and after the onset of MCD and the motoneurons in their lateral motor columns counted. Their results showed definitively that the increased number of motoneurons in the acceptor spinal cord was the result of reduced degeneration rather than increased proliferation. Thus, according to Hamburger, the apparent "hyperplasia" found after increase of the peripheral target is technically a "hypothanasia" (Hamburger, 1980).

A similar study was performed in the chick embryo by O'Brien and Oppenheim (1990). However, rather than transplant the limb bud to the lumbar cord, they placed it over the thoracic cord. They thus examined the effect of increased target size by ectopic tissue, that is, tissue that is not normally innervated by that spinal region. O'Brien and Oppenheim found that cell death was in fact reduced by the increase in target size. After the period of MCD, however, the foreign muscle atrophied and the rescued motoneurons degenerated. Which was the cause and which the effect was undetermined.

The creation of supernumerary and ectopic limbs has also been performed in amphibians. These studies, however, were ambiguous as to the relationship between increase in muscle size and the course of MCD. On one hand, the addition of supernumerary limbs in the frogs *Xenopus laevis* and *Rana pipiens* was found to produce an increase in motoneurons of the corresponding spinal cord (Hollyday and Mendell, 1976; Maheras and Pollack, 1985). On the other hand, studies in the same species showed no effect of limb bud transplantation on MCD (Pollack, 1969; Rubin and Mendell, 1980, Harrison, 1989).

3.3.1.2 Decreased Target Muscle Size / Increased MCD

The first demonstration of a relationship between a reduction in peripheral muscle and a concordant reduction in the number of motoneurons was shown in 1909 by Shorey. Her study showed that the removal of a limb bud from the chick embryo resulted in marked hypoplasia of the spinal motor column. As mentioned previously, this study was followed up by Hamburger (1934) and demonstrated a specific relationship between the proportion of muscle removed and the degree of motor hypoplasia. Additionally, Cowan and Wenger (1967) showed hypoplasia in the trochlear nucleus after extirpation of the chick optic vesicle. Other studies in the 1960's on frog limb innervation showed similar hypoplasia in the absence of target tissue (Hughes, 1962; Prestige, 1967). Yet, as with motor hyperplasia, it was unknown if alteration of MCD was responsible for the fewer motoneurons.

In 1978, Oppenheim and co-workers investigated cell death of motoneurons in the chick embryo spinal cord after limb bud removal. This study used the same animal model as Hamburger did for hyperplasia and followed similar experimental paradigms. In this manner, they showed that in the absence of a limb bud approximately 90% of lateral motor column neurons degenerated after a normal period of neurogenesis. This decline was greater than the unoperated side which exhibited only 50% motoneuron loss. More importantly, all the additional loss on the operated side occurred within the normal period of MCD and was not the result of secondary degeneration.

Numerous studies over the past decade have reinforced Oppenheim's findings that peripheral deprivation causes enhanced degeneration during the period of normal cell death (Laing, 1982; Whitelaw and Hollyday, 1983; Lanser et.al., 1986). In a "natural" experiment of peripheral deprivation the spinal cord of the limbless mutant chick embryo was studied. This mutant begins to develop limbs but at approximately day 3 of gestation the mesoderm degenerates and is completely gone by day 4. This is several

days before the period of naturally occurring cell death. In these mutants, 85% of motoneurons were lost during MCD as opposed to 40% in normal embryos (Lanser and Fallon, 1984; Lanser and Fallon, 1987).

Although these studies support the theory of target dependent MCD, they failed to differentiate between the effects of muscle and the effects of other limb tissue. In 1991, Phelan and Hollyday showed conclusively that it was muscle and not other tissues in the limb bud that influenced MCD. They removed only muscle precursor cells from the wing somites of chick embryos. As with removal of the whole limb, motoneuron degeneration was accelerated and increased within the period of normal MCD.

Although thoroughly studied in birds and amphibia, investigations into the occurrence and factors affecting MCD in mammalia have been few. The principle reason has been the difficulty in performing prenatal surgery *in utero*. As such, a group in Australia has turned to marsupials (*Macropus eugenii*) which undergo late embryonic and fetal maturation in an easily accessible maternal pouch (Comans et.al., 1987; Comans et.al., 1988). They studied the effects of radical or partial limb bud removal on motoneuron development in the lateral motor column of the lumbar spinal cord. Their results demonstrated increased MCD with target deprivation. In addition, in limbs in which all muscle was successfully removed, virtually no motoneurons were apparent after the period of MCD. Thus, the general characteristics of MCD appear to be conserved across amphibian, avian, and mammalian classes.

3.3.2 Muscle Function

Although a dependence of developing neurons on their target muscle was clearly demonstrated, the mechanism and nature of the epigenetic signals was far from understood. This initiated a series of experiments directed at elucidating the mode of signal transduction. Evidence began to mount that cell death was occurring at or just after innervation of the target (Hughes, 1968; Rogers and Cowan, 1973; Landmesser and

Pilar, 1974; Hamburger, 1975). Further investigation showed that all motoneurons, even those destined to die, innervated the target tissue (Prestige and Wilson, 1972; Prestige and Wilson, 1974; Chu-Wang and Oppenheim, 1978b). This led to studies exploring the role of the neuromuscular synapse in regulating MCD. These studies showed that the function of the synapse, and therefore activity of the muscle play an important role in the survival of differentiating motoneurons. As with muscle size, a basic tenet dictated the relationship between muscular activity and the resultant cell death: 1) if target muscle activity is decreased, motoneuron cell death is increased and 2) if target muscle activity is increased, motoneuron cell death is decreased.

3.3.2.1 Decreased Muscle Activity / Decreased MCD

To understand if synaptic mechanisms play a role in MCD the muscles of chick embryos were immobilized with neuromuscular blocking agents (Pittman and Oppenheim, 1978; Laing and Prestige, 1978; Pittman and Oppenheim, 1979). These included curare, α -cobratoxin, α -bungarotoxin and botulinum toxin. All these agents successfully produced a cessation of spontaneous neuromuscular activity in the developing chick limb. When the lumbar spinal cord was examined, motoneuron counts were elevated when compared with saline injected controls. In fact, the number of surviving motoneurons was even greater than that seen when a supernumerary limb was transplanted (Hollyday and Hamburger, 1976). Furthermore, if blocking agents were administered after the period of normal cell death, no change in motoneuron numbers was observed. These findings indicated that the occurrence of MCD is related to the activity of the innervated muscle during the period of natural cell death. Specifically, a reduction in neuromuscular activity leads to a reduction in MCD.

The use of the chick embryo spinal cord model has reproduced the above findings many times (Zilles et.al., 1981; Oppenheim and Bursztajn, 1983; Oppenheim, 1984; Okada et.al., 1989; Oppenheim et.al., 1989). Additionally, neuromuscular blockade has

been shown similarly to affect the course of MCD in other neural centers and in other species. For example, tubocurare successfully prevented cell death in the trigeminal motor nucleus of the chick embryo (Arens and Straznicky, 1986). Furthermore, α -bungarotoxin has been shown to prevent MCD in the trochlear nucleus of the duck embryo (Creazzo and Sohal, 1979). Similar results were seen in *Xenopus* after the introduction of α - and β -bungarotoxin (Olek, 1980).

As mentioned previously, eutherian embryos are difficult to use in experimental manipulations of neuromuscular development. However, as opposed to limb bud removal and transplantation, the production of neuromuscular blockade is relatively simple. This is due to the efficacy of these agents in producing paralysis upon introduction into the amniotic sac alone. Using this technique, Harris and McCaig (1984) were able to prevent naturally occurring cell death in the cervical and thoracic spinal cord of prenatal rats. The same result was also produced in the lumbar spinal cord of the developing mouse (Houenou et.al., 1990).

The absence of activity in prenatal muscle can also be found naturally. For example, the mouse mutant, muscular dysgenesis, exhibits defects in muscle maturation including the absence of prenatal muscular contractions (Pai, 1965a; Pai, 1965b). This strain represents a natural model for the investigation of peripheral influence on central neuronal differentiation. In 1986, Oppenheim and co-workers compared motoneuron development in muscular dysgenesis as compared to normal controls. As with artificial paralysis, mutant mice showed a reduction in the amount of cell death in the lumbar spinal cord. In fact, only motoneurons were affected as ganglia cell death in mutants and normals was the same. Additionally, results showed that neurogenesis and differentiation were unaffected in the mutant strain. Thus, the genetic defect is restricted to muscle development and changes in MCD must be due to epigenetic signals from the target tissue.

3.3.2.2 Increased Muscle Activity / Increased MCD

Although the above experiments and observations indicate that muscle activity affects MCD, the relationship appears to be more complicated. Hexacarbacholine produces muscular paralysis, however, has no effect on the survival of motoneurons in the chick spinal cord (Oppenheim and Maderdrut, 1981). This may be the result of a different neuromuscular blocking mechanism, i.e., hexacarbacholine is a depolarizing neuromuscular blocking agent. In order to explore further the effect of muscle activity, experiments were designed to increase the prenatal motility of developing muscles. This would indicate if indeed some aspect of activity was responsible for the modification of MCD.

The first attempt at increasing muscle activity in the prenatal chick employed the use of neuromuscular agonists (Oppenheim and Maderdrut, 1981). Using the same techniques as for neuromuscular blockade, nicotine, carbachol, and choline were administered to the chick embryo at the time of naturally occurring cell death. MCD was found to be increased with the administration of carbachol. However, no change was evident with nicotine or choline. In the same experiment, the cholinesterase inhibitor, eserine, was found to produce significant increases in naturally occurring cell death. The results were, therefore, inconclusive as to the effect of increased activity on MCD.

In order to clarify the above results, another method of increasing neuromuscular activity was employed, direct electrical stimulation (Oppenheim and Núñez, 1982). In short, electrodes were implanted into the legs of chick embryos and stimulated during the period of normal cell death. Compared to unstimulated controls, the number of motoneurons in the lateral motor column was decreased by approximately 20%. In addition, there was no change in the course of normal cell death in the spinal ganglia. Thus, increased muscle activity, like decreased activity, specifically affects motoneurons by influencing the patterns of MCD.

3.3.3 Afferent Neurons

The focus of much of the research into developmental cell death in neuromuscular systems has been the interplay between the motoneuron and its target. However, over the past decade evidence has begun to show that afferent input is an important determinant of developmental survival in many neuronal systems (Parks, 1979; Cunningham, 1982; Clarke, 1983; Furber and Oppenheim, 1984; Sohal et.al., 1990; Clarke, 1992).

In order to address the possibility that afferent neuronal input plays a role in MCD, Okado and Oppenheim (1984) investigated the effect of deafferentation of motoneurons in the chick embryo spinal cord. This study eliminated primary afferent input and descending corticospinal input, both individually and simultaneously, prior to the onset of MCD. Additionally, in order to distinguish between the effects of afferent feedback and muscle feedback, some embryos were also paralyzed with curare. The results showed that individually, descending and primary afferent deprivation had no effect during the period of normal MCD, but did result in degeneration at later stages. However, the removal of both types of afferent input caused 37% more cell death than found in controls. Furthermore, curare was unable to rescue deafferentation sensitive motoneurons. Thus, these results showed that afferent input to motoneurons also exerts an influence on the course of MCD in the developing organism.

3.4 Mechanisms of MCD

3.4.1 Requirements for Epigenetic Influence

The influence of the periphery over central motoneuronal development has been clearly demonstrated. However, the mechanism by which target muscle or afferent neurons dictate the course of MCD is unknown. Research during the past two decades has found at least two factors involved in the developmental feedback between target and

motoneuron. The first is the requirement for synaptic contact between nerve and muscle and the second, the availability of neuronotrophic factors produced by the target tissue.

3.4.1.1 Synaptic Contacts

During the late 1960's and early 1970's studies demonstrated a temporal relationship between the onset of neuronal death and the innervation of the target (Hughes, 1968; Rogers and Cowan, 1973; Landmesser and Pilar, 1974). This led to suggestions that neurons compete for a limited number of synaptic sites (Hamburger, 1975; Landmesser and Pilar, 1978). Those motoneurons which are able to establish contact with the target muscle, or which are themselves contacted by afferent neurons, would survive. Non-synapsing motoneurons would degenerate. It was not clear, however, how synaptic contact could lead to survival and it was suggested that the signaling mechanism was related to activity at the neuromuscular junction (Changeux and Danchin, 1976).

As described above, blockade of synaptic transmission with several neuromuscular antagonists (Pittman and Oppenheim, 1978) led to decreased cell death. These findings seemingly contradicted the contention that contact with an active synapse was necessary for survival. In fact, they indicated that inactive synapses prevent cell death leading to the suggestion that active synapses may actually cause cell death. This latter hypothesis was supported by findings of increased cell death with enhanced neuromuscular activity (Oppenheim and Maderdrut, 1981; Oppenheim and Núñez, 1982).

In order to resolve the counter-intuitive effects of neuromuscular blockade on MCD, studies looked at the effects of such manipulations on the motoneuron. Initial studies showed no differences between motoneurons rescued from death and those surviving in controls (Oppenheim, 1981; Oppenheim, 1984; Oppenheim et.al., 1989). However, there have been changes found in the patterns of muscle innervation in pharmacologically paralyzed muscles that may be responsible for the prevention of cell death. In paralyzed muscle, the developing nerve fibers branch more (Dahm and Landmesser, 1988) and

show more polyneuronal innervation at an increased number of neuromuscular junctions (Ding et.al., 1983; Oppenheim, 1984; Dahm and Landmesser, 1991). Furthermore, muscle fibers treated with curare manifest a greater number of acetylcholine receptors (Oppenheim, 1991). More importantly, paralytic agents which do not affect cell death, do not elicit increases in these receptors (Oppenheim, 1989). Since acetylcholine receptors are part of the neuromuscular junction, these additional receptors may define additional synapses which can promote neuronal survival (Oppenheim, 1991). Thus, research during the past decade has explained results which seemed contrary to the synaptic theory and at the same time provided evidence in its support. However, although it appears neurons need synaptic contact to survive, it is still unclear what processes at the synapse lead to the prevention of cell death.

3.4.1.2 Trophic Factors

As early as 1909, the dependence of developing neurons on their target tissue was clearly demonstrated (Shorey, 1909). However, the nature of this dependence remained a mystery until the discovery and subsequent characterization of nerve growth factor (NGF) (Hamburger and Levi-Montalcini, 1951, 1953; Levi-Montalcini, 1982). From these findings arose a theory to explain the development of neuron-target interactions, the neurotrophic theory (Purves, 1986; Adler, 1988; Oppenheim and Haverkamp, 1988; Oppenheim, 1989). This theory postulates that target tissues produce limited amounts of trophic factors necessary for neuronal survival. The innervating neurons compete for these substances and those gaining sufficient access are then able to survive and differentiate normally. Those neurons obtaining insufficient quantities of trophic factor degenerate. The demonstration of naturally occurring motoneuron death (Cowan, 1973; Hamburger, 1975) has initiated efforts to find a putative motoneuron trophic factor.

The first substance investigated for its possible role as a motoneuron trophic factor was nerve growth factor, NGF. Initial experiments focused on the ability of

motoneurons to acquire NGF from target tissue. Stöckel and co-workers (1975) showed that in the adult rat, NGF was taken up by sensory neurons and sympathetic adrenergic neurons but not by cholinergic motoneurons. It was subsequently found that *embryonic* motoneurons also lacked the ability to take up NGF (Brunso-Bechtold and Hamburger, 1979). In terms of survival promoting ability, NGF was shown to be active on dorsal root ganglion cells and sympathetic neurons (Gorin and Johnson, 1979; Hamburger et.al., 1981). However, NGF failed to rescue motoneurons from naturally occurring cell death in the chick embryo (Oppenheim et.al., 1982). Thus, these findings show no neurotrophic capabilities for NGF during neuromuscular development. Further studies, however, revealed suprising results.

A study of NGF receptor expression during chick embryogenesis showed binding in classic NGF target sites (i.e., sympathetic and dorsal root ganglia). Furthermore, an unexpected transient expression of NGF receptor was found in spinal cord motoneurons and skeletal muscles (Raivich et.al., 1985). This expression was early in development and corresponded with the period of MCD. Subsequent investigation showed similar expression and temporal patterns in higher motor centers, including the glossopharyngeal nerve (Raivich et.al., 1987). Based on these findings, the uptake of NGF by embryonic motoneurons was reexamined but at these earlier stages of development (Wayne and Heaton, 1988; Wayne and Heaton, 1990). It was found that during the period of MCD, and only during this period, motoneurons were capable of taking up NGF. The fact remained, however, that NGF did not influence the course of MCD or promote motoneuron survival. It has been postulated that the concurrent expression of NGF receptor on muscle and motoneurons during the period of MCD, and thus synaptogenesis, may promote cell-cell interactions and play a role in the formation of neuromuscular junctions (Wayne and Heaton, 1990).

Recent studies have examined the effect of other growth factors on developing motoneurons. One of these, basic fibroblast growth factor (bFGF) appears to be involved

in the induction of embryonic tissue (Rifkin and Moscatelli, 1989). Additionally, bFGF has been shown to promote central and peripheral neuronal development *in vitro*, including the development of cholinergic neurons (Ferrari et.al., 1989; Henderson, 1988). However, when studied *in vivo*, bFGF had no effect on motoneuron survival (McManaman et.al., 1990). These results indicate the importance of *in vivo* studies for the assessment of motoneuronal trophic effects. Further illustrations of this point are recent studies on ciliary neurotrophic factor (CNTF). CNTF has been shown to support the survival of sensory and autonomic neurons *in vitro* but not to prevent their naturally occurring cell death *in vivo* (Oppenheim et.al., 1991). Even more importantly, CNTF has no effect on motoneurons in culture but does prevent MCD *in vivo* (Wewetzer et.al., 1990; Oppenheim et.al., 1991). Thus, CNTF may act as a motoneuron trophic factor, but cannot be characterized by standard *in vitro* assays.

In addition to CNTF, transforming growth factor β_1 (TGF β_1) has been proposed as a motoneuron trophic factor, although its activity was discovered *in vitro* and has not yet been shown *in vivo* (Martinou et.al., 1990). Recent reports also ascribe motoneuron survival activity to brain-derived neurotrophic factor (BDNF) and neuroleukin (Gurney et.al., 1986; Oppenheim et.al., 1992; Schecterson and Bothwell, 1992). Current investigations are continuing to characterize the interactions between motoneurons and these substances as well as with the recently discovered neurotrophin-3 (Kuno, 1990).

Rather than test previously characterized substances, many investigators have attempted to find motoneuron survival factors in target muscle. These studies have shown that target muscle does indeed contain trophic factors. For example, medium conditioned with muscle cells is capable of promoting the growth of spinal neurons in culture (Henderson et.al., 1981; Nurcombe et.al., 1984; Smith et.al., 1986).

Additionally, in experiments using embryonic muscle extract, embryonic motoneurons were successfully kept alive in culture (Dohrmann et.al., 1986; O'Brien and Fischbach, 1986). As noted above, differences between cultured and *in vivo* neurons requires the

demonstration of neurotrophic activity in the developing organism. Indeed, it was demonstrated that muscle extracts can rescue developing motoneurons from natural cell death *in vivo* (Oppenheim et.al., 1988; Petruzzelli and Hughes, 1989).

Based on muscle extract experiments, McManaman et.al. (1990), were able to purify a novel neurotrophic factor from rat skeletal muscle. This factor increases the levels of choline acetyltransferase (ChAT) in cultured motoneurons and was therefore named ChAT development factor (CDF). When they applied CDF to developing chick embryos MCD was significantly decreased as compared to normal controls. Furthermore, CDF affected only motoneurons as sensory and sympathetic neurons underwent normal levels of natural cell death. Thus, the finding of a specific motoneuron trophic factor provides strong evidence in support of the neurotrophic theory.

It should be noted that in certain regions of the nervous systems hormones have been shown to act as trophic factors in influencing patterns of MCD. This most clearly occurs in the sexually dimorphic motor nuclei of the rat (Sengelaub and Arnold, 1989; Sengelaub et.al., 1989a; Sengelaub et.al., 1989b). The dorsomedial and dorsolateral nuclei of the lumbar spinal cord innervate specific muscles of the perineum. These muscles form in both genders but early in development atrophy in the female. During the first postnatal week there is a period of cell death in the corresponding motor nuclei during which females loses many more motoneurons than males. It was demonstrated that the excess death in females may be prevented by the addition of male steroids. Hormones have not been shown to produce differential cell death in non-sexually dimorphic motor nuclei.

3.4.2 Mechanism of Epigenetic Influence

The neurotrophic theory contends that competition for trophic factors during specific periods of development determines patterns of cell death. This implies to some that trophic factors are in limited quantities, thus ensuring that a proportion of the innervating

population will be unable to obtain the sufficient amounts required to survive (Oppenheim and Haverkamp, 1988; Oppenheim, 1989; Kuno, 1990). This, however, does not necessarily explain the effects of peripheral manipulations on the course of MCD. For example, when limb muscles are paralyzed with neuromuscular blocking agents MCD is reduced. This would indicate an increase in the amount of trophic factor produced by the immobilized muscle. It was shown, however, that paralyzed muscle contains no more neurotrophic factor than normal muscle (Tanaka, 1987).

Failure of the neurotrophic theory to explain all experimental results adequately has led to the counterproposal that trophic factors are, in fact, in relative abundance but that access to these substances is limited by axonal branches and synaptic sites (Landmesser and Pilar, 1978; Dahm and Landmesser, 1988; Oppenheim, 1991). This limited access has been postulated to account for the changes in MCD observed during neuromuscular blockade (Landmesser, 1992). For example, as described above, paralysis of developing muscle promotes axonal branching and the formation of additional synaptic sites. This increase in synapses would allow more motoneurons to establish synaptic contact and thus obtain access to the trophic factors accounting for the increased survival.

Additionally, the effects of muscle size on MCD could be explained by the increase or decrease in number of myofibers and thus potential synaptic contacts. This, in turn, would allow more or fewer axons access to the trophic factors. The "access" hypothesis, however, does not completely explain all the experimental findings. For example, the addition of exogenous trophic factor can rescue motoneurons from dying. Yet, it has not been shown that additional trophic factor influences the number of synaptic sites.

At the present time, no one theory adequately explains all the observed effects of muscle size and function on MCD. Further investigation into the nature of trophic factor production and uptake are necessary to elucidate the mechanism by which epigenetic signals from peripheral tissue can influence the survival of innervating motoneurons.

3.4.3 Signal Transduction and Genetics of MCD

It has been shown that neuronal response to the size and function of target tissue is in large part a response to the neurons ability to acquire trophic factors. Those neurons with sufficient access to the trophic factors survive and differentiate, while those with limited access die. The means by which trophic factors act upon the cells is not entirely clear, although recent studies on motoneurons indicate that trophic factors can influence gene expression. For example, levels of choline acetyltransferase and calcitonin gene related peptide in motoneurons are altered by treatment with curare during the period of MCD (Esquerda et.al., 1989; Haverkamp and McManaman, 1990). It has been proposed that these changes are related to the effects of trophic factors on the genome. Recent investigations have focused on the cellular and molecular processes induced by trophic factors in an effort to explain the survival or degeneration of developing neurons.

In order for trophic factors to influence genetic expression there must be a pathway for signal transduction. This may involve direct transport to the nucleus, as seen with hormones, or make use of a second messenger. Evidence for the latter has been demonstrated by the effects of dibutyryl-cyclic GMP (db-cGMP) on chick embryo MCD (Weill and Greene, 1990). This study found that the addition of an unhydrolyzable analog of cGMP to the developing chick prevented the occurrence of natural motoneuron death in the spinal cord. From this data, it was postulated that trophic factor binding at the synaptic site activates a guanylate cyclase which, in turn, produces cGMP. The cGMP is then retrogradely transported to the soma where it mediates gene expression. Additional evidence in support of this theory, is the natural rise of cGMP levels in ciliary ganglion cells observed during the period of cell death (Weill, 1986).

Further evidence for a second messenger transduction system in mediating trophic factor activity was provided in experiments on cultured sympathetic neurons maintained by NGF (Martin et.al., 1990). In brief, this study showed that treatment with AraC, a deoxycytidine (dC) analog, caused the death of NGF supported neurons, not directly, but

by mimicking NGF deprivation. The authors postulated that *araC* prevented the second messenger produced after NGF binding from acting on the genome. Furthermore, they proposed the existence of two genetic programs: a trophic program and a death program. The trophic program expresses genes necessary for neurotransmitter production and neurite outgrowth. The death program expresses genes initiating apoptosis and causing cell death. Thus, at specific stages of development, the neuron becomes dependent on exogenous trophic factors to maintain repression of the death program. Failure to gain access to these factors results in derepression of the death program and expression of "suicide" genes.

Over the past several years, evidence has been gathered in support of the existence of suicide genes and their products. For example, as noted apoptosis exhibits distinct patterns of chromosome cleavage induced by specific endonucleases. These endonucleases are dependent upon high levels of calcium (McConkey et.al., 1990). It has been demonstrated that somatostatin, a hormone that reduces intraneuronal calcium levels, can prevent MCD in the chick embryo spinal cord presumably by blocking endonuclease activity (Weill, 1991).

Further evidence in support of a death program is the finding that inhibitors of RNA and protein synthesis can prevent natural cell death induced by trophic factor deprivation (Martin et.al., 1988). This indicates that cell death is an active process requiring the expression of specific genes and subsequent translation of suicide proteins. Furthermore, it supports the hypothesis that trophic factors not only induce differentiation, but actively repress degeneration. The same finding was reproduced *in vivo* where treatment of chick embryos with RNA and protein synthesis inhibitors reduced the amount of MCD in spinal motoneurons both naturally and after limb bud extirpation (Oppenheim, et.al., 1990).

Definitive proof of the existence of suicide genes has come with the recent discoveries of specific genes in the nematode and moth responsible for naturally

occurring cell death. The nematode, *Caenorhabditis elegans*, is a highly characterized model for the study of developmental cell death (Schwartz, 1991). Although cell death in the nematode is different than MCD in that no epigenetic factors have been found, it serves as a good model for degenerative mechanisms. It is known that two genes, *ced-3* and *ced-4*, are essential for the occurrence of natural cell death (Ellis and Horvitz, 1986). Recently, another gene has been found which regulates the activity of these genes, *ced-9* (Hengartner et.al., 1992). Two mutations were isolated, one in which *ced-9* is inactive and one in which it is always active. Inactive *ced-9* causes cells which normally survive to undergo natural cell death. Constitutive *ced-9*, on the other hand, rescues cells which normally die. It is unclear what signals *ced-9* to regulate the functions of *ced-3* and *ced-4*, but in higher organisms, these signals may take the form of trophic factors.

Far more relevant to vertebrate cell death is the finding of genes encoding cell death proteins in the tobacco hornworm moth, *Manduca sexta*. In the larva, specific muscles undergo metamorphic cell death in response to a decline in the level of the hormone, 20-hydroxyecdysone. From these muscles several genes were cloned which express during cell death and are responsive to levels of the ecdysteroid (Schwartz, 1991). One of these genes has been determined to be polyubiquitin. It contains nine ubiquitin coding regions which upon expression causes rapid elevation of free cytoplasmic ubiquitin. Although found in all cells, high levels of ubiquitin may function in proteolysis during the degenerative process. Furthermore, the author indicates that levels of ubiquitin appear to rise in the chick embryo nervous system at the time of MCD.

Taken collectively, these studies indicate a mechanism by which trophic factors produced by target tissue may influence the survival of innervating neurons. It may be postulated that at the synaptic site trophic factors bind to neurons and induce the formation of a second messenger, possibly cGMP. This messenger is retrogradely transported to the soma where it stimulates the expression of the trophic program. The trophic program transcribes genes which promote neurotransmitter production and

neurite outgrowth. In addition to inducing expression, the second messenger, by an araC sensitive process, would repress the expression of the death program. In cells that fail to acquire sufficient amounts of trophic factor, the death program is derepressed. This would lead to the production of "suicide" proteins which may include specific endonucleases and multiple copies of ubiquitin. These proteins, in turn, would actively promote the degradative changes observed in apoptosis.

3.5 The Role of MCD

The previous sections have demonstrated the dependence of MCD on signals arising from the target tissue, afferent neurons, and even the external environment. Thus, the course of MCD may be altered by developmental abnormalities in peripheral tissue as well as extra-uterine teratogens. However, during *normal* development, the extra-uterine environment and the epigenetic signals exhibit much less variation than in the dramatic experimental manipulations heretofore described. As such, most individuals within a species undergo similar courses of MCD and have comparable numbers of motoneurons. This is demonstrated, particularly in the chicken, by the many studies in which the course of MCD has been consistently reproduced in non-experimental control organisms (see review, Oppenheim, 1991). What, therefore, in the framework of a normal gestational milieu, is the role of MCD?

It has been postulated that MCD serves to establish a quantitative matching of motoneurons and their target tissues (Lamb, 1984; Oppenheim, 1985; Oppenheim, 1991). Quantitative matching refers to the innervation of a specific number of muscle fibers with an appropriate complement of motoneurons. This ratio of muscle fibers to number of motoneurons defines the "motor unit" which largely determines the specificity with which a muscle may be controlled (Windhorst et.al., 1989). Thus, MCD is thought to reduce the initial overproduction of motoneurons to an appropriate number for the size of

the innervated muscle. This notion of quantitative matching has been supported by the large body of research described previously on the effects of target size. Moreover, experiments designed specifically to demonstrate a quantitative relationship between MCD and the number of muscle fibers support this theory (Tanaka and Landmesser, 1986; Habgood et.al., 1989).

Though the quantitative matching theory provides an explanation for the amount of cell death observed, it does not explain the reasons for the initial overproduction of motoneurons. One hypothesis is that mitotic constraints at the ependymal layer prevent the production of the proper number of motoneurons (Oppenheim, 1985). That is, precursor neurons may be genetically determined to undergo a specific number of mitotic divisions which would double the number of motoneurons with each division. For example, as postulated by Oppenheim (1985), if there are 10,000 precursor motoneurons and the requirement for that muscle is 13,000, the next division would result in 20,000 cells. Thus, MCD would be a natural result of a genetically predetermined pattern of mitoses.

Experiments that have shown a dependence of MCD on target size, although supporting the quantitative theory, have failed to demonstrate an exact relationship between muscle fibers and motoneuron numbers (Hollyday and Hamburger, 1976; Lamb, 1980). As such, an alternative, or additional role for MCD has been proposed which maintains that MCD serves to eliminate errors in axonal projections (Landmesser and Pilar, 1978; Lamb, 1984; Oppenheim, 1985; Oppenheim, 1991). Central to this theory is the contention that neurons do indeed make errors in their projections. However, several studies have show that axonal projections, especially those of motoneurons, are exceptionally accurate (Landmesser, 1980; Oppenheim, 1981; Whitelaw and Hollyday, 1983; Lance-Jones, 1984; Tosney and Landmesser, 1985). Furthermore, when motoneurons were forced to innervate a foreign muscle they were not eliminated by MCD (Landmesser and O'Donovan, 1984).

It was proposed that less dramatic errors, such as improper synaptic patterns, may form between motor axons and their targets. Such errors were found to be relatively common in other neuronal centers and were eliminated by neuronal death (O'Leary et.al., 1986; Catsicas et.al., 1987; Nakamura and O'Leary, 1989; Clarke, 1992). Investigations into the role of MCD in eliminating small scale errors focused on the fate of primitive axonal projections. These motoneurons contact the target tissue before it is properly formed and thus establish a diffuse and improper pattern of innervation (Lamb, 1984). Studies of these motoneurons in the frog and chick showed that they are eliminated shortly after the onset of MCD most likely by degeneration (Lamb, 1977; Laing, 1982). These studies provide initial evidence that the elimination of improper synapses may be a role of MCD during neuromuscular development. MCD may also serve to eliminate motoneurons projecting to the wrong muscle fiber type or motoneurons contacted by the wrong afferents (Lamb, 1984).

Proper neuromuscular control relies upon well defined motor units with specific motoneuron connections. Furthermore, corticospinal and afferent neurons must contact the correct motoneurons for eliciting the desired response. This specificity is established, in large part, by MCD in its response to epigenetic signals from the target and other neurons. As such, MCD serves to define patterns of innervation and thus plays a critical role in the development of neuromuscular control.

3.6 Animal Models

Over the past two decades the epigenetic factors affecting the course of MCD have been extensively studied. As previously mentioned, the most commonly used model for these studies has been the chick embryo spinal cord. As such, the course of MCD in other species and other motoneuronal populations has not been well characterized. The following tables demonstrate the lack of investigation of MCD in mammals and higher

motoneuronal centers (also see review, Oppenheim, 1991). In particular, note the absence of studies in the motor nuclei innervating the upper respiratory tract.

3.6.1 Animals Studied

While several different mammals have been examined for the occurrence of MCD, the overall number of studies represented is relatively low. For example, few studies have investigated MCD in rats (Harris and McCaig, 1984; Oppenheim, 1986) and mice (Zilles and Wingert, 1973; Lance-Jones, 1982; Ashwell and Watson, 1983; Houenou et.al., 1990) while numerous studies have employed the chicken (see section 3.3). Table 3.1 lists those animals in which MCD has been examined including studies of specialized motoneurons found in fish, i.e., electromotor neurons and primary motoneurons (Fox and Richardson, 1984; Eisen et.al., 1990). Additionally, although Oppenheim (1991) indicated that MCD has been studied in monkeys, the original study has not been located.

3.6.2 Motor Nuclei Studied

Motoneuron cell death occurs in somatic, branchiomeric, and visceral motoneuron populations. The most widely studied have been somatic motoneurons of the spinal cord and visceral motoneurons of the sympathetic ganglia. The only studies of branchiomeric motoneurons are in the trigeminal (first arch) (Arens and Straznicky, 1986) and facial (second arch) motor nuclei (Ashwell and Watson, 1983). No studies have examined MCD as it pertains to the motor pools of the third, fourth and sixth branchiomeric arches. Specifically, MCD in the nucleus ambiguus, that nucleus innervating the larynx and pharynx, has not been studied in any species. Furthermore, only one study has described cell death in the hypoglossal nucleus (Milutinovic et.al., 1992). This study focused on the derivation of a mathematical model for developmental regressions and provided an overview of cell death in several nuclei. As such, little information was provided as to the course of MCD in XII.

Table 3.1**Animals In Which Motoneuron Cell Death Has Been Studied**

Fish	Amphibians	Birds	Mammals
electric fish zebrafish European eel	frog turtle salamander	duck quail chicken	rat mouse wallaby opossum monkey human

Table 3.2
Nuclei And Animals In Which Motoneuron Cell Death Has Been Studied

Somatic and Branchiomeric Motoneurons

Nucleus	Animal
Spinal Motoneurons	fish frog turtle chicken quail mouse rat opossum wallaby monkey human
Electromotor Nucleus	electric fish
Primary Motoneurons	zebrafish
Oculomotor Nucleus (cranial nerve III)	duck chicken mouse
Trochlear Nucleus (cranial nerve IV)	salamander frog chick quail duck human
Abducens Nucleus (cranial nerve VI)	duck
Trigeminal Motor Nucleus (cranial nerve V)	chicken
Facial Nucleus (cranial nerve VII)	mouse
Hypoglossal Nucleus (cranial nerve XII)	human

Autonomic Motoneurons

Neuronal Population	Animal
Ciliary Ganglion (postganglionic parasympathetics)	chicken
Sympathetic Chain Ganglion (postganglionic sympathetics)	chicken rat
Sympathetic Cells (preganglionic sympathetics)	chicken
Dorsal Motor Nucleus of the Vagus (preganglionic parasympathetics of cranial nerve X)	chicken

Chapter 4

URT and Brainstem Anatomy of the Rat

Chapter 4 and the remainder of this thesis will present original work on the development of URT motor nuclei in the rat. This chapter, will detail selection of the rat as the choice of animal model and provide background in rat URT and brainstem anatomy necessary for analysis and interpretation of the experimental studies. All dissections and histological preparations heretofore presented are the work of the author except where noted.

4.1 Choice of Animal Model

The goal of this study was to define prenatal periods critical to the development of URT neuromuscular control. This was accomplished by investigation of developing brainstem motor nuclei which innervate muscles of the URT. As such, consideration of an animal model focused on use in a developmental neuroanatomical study, and use in a study of the URT. The animal that best accommodated these criteria and was chosen for the present study was the rat.

The rat central nervous system has been finely detailed in atlases defining distinct anatomical and biochemical regions of the rat brain (Pellegrino et.al., 1979; Paxinos and Watson, 1982; Mugnaini and Oertel, 1985). This has made the rat nervous system accessible to all researchers and, as such, the rat brain has been the focus of many neurobiological studies (e.g., Armstrong et.al., 1983; Young and Stanton, 1991; Fabri and Burton, 1991; Hay-Schmidt and Mikkelsen, 1992; Van Bockstaele and Aston-Jones, 1992; Laird and Bennett, 1992; Larkman and Kelly, 1992; Machado and Bonagamba, 1992; Caicedo and Herber, 1993; Rhoades et.al., 1993; Sun and Cassell, 1993). In addition, the rat brain has been used to explore developmental change, including maturation of the brainstem and its neuronal centers (e.g., Altman and Bayer, 1980; Kubota et.al., 1988; Gomez et.al., 1990; Phelps et.al., 1990; Friedman et.al., 1991; Grothe et.al., 1991; Lasiter and Kachele, 1990; Ashwell and Waite, 1991; Chiaia et.al., 1992). The extensive research into the rat brain and brainstem has established the rat as a reliable and well-characterized model for neurobiological investigation.

Another concern with regard to the animal model was its ability to serve as a template for defining normal neuromuscular development in the mammalian URT. Thus, the animal needed to exhibit a representative mammalian URT with little specialization in morphology or function. The rat was chosen as it exhibits a larynx positioned high in the neck creating largely separate pathways for respiration and deglutition (Reidenberg,

1988). As noted in Chapter 1 (figure 1.1), this morphology, and associated patterns of respiration and deglutition, are found in all terrestrial mammals and represent the baseline mammalian condition.

The specific methods used in this study imposed additional requirements on the choice of animal model. For example, this study employed quantitative methods to assess neuronal populations at many developmental stages and thus required a large sample size. The rat has large litters and thus allowed many specimens to be collected from a single mother. Further, as this study largely investigated prenatal and early postnatal ages, an animal with a short gestational period able to be examined at specific developmental stages was necessary. The rat gestational period is only 22 days, and timed-pregnancy females were readily available from commercial vendors. As this study primarily focused on the third gestational week, the use of a commercial vendor greatly reduced the time and cost of this experiment relative to breeding rats "in house". Finally, brainstems and cranial nerves had to be of sufficient size so as to be dissected and manually manipulated, particularly at early gestational stages. While the mouse fit most of the experimental criteria, this last condition made the rat more suitable to the current experiment.

4.2 The Rat Upper Respiratory Tract

As described above, an important condition in the choice of animal model was that the morphology of the URT be representative of the basic mammalian pattern. That is, the animal should have an aerodigestive tract with the larynx positioned high in the neck such that the epiglottis and soft palate are opposable and pathways for respiration and deglutition are largely distinct. The rat exhibits such a URT morphology which can be demonstrated on gross and histological examination. Further, histological observation of developing rats demonstrates the origins of such a URT morphology. The figures at the

end of this chapter will serve to familiarize the reader with the size and morphology of the developing rat and the morphology of the URT.

4.3 The Brainstem

Although this study explored many developmental phenomena in the rat brainstem, all data were gathered from transverse sections of the lower medulla. Thus, familiarity with the anatomy of the rat brainstem, particularly at prenatal ages, is important in interpreting the experimental results. The following figures will serve to familiarize the reader with the anatomy of the rat brainstem, in particular, the locations of the nucleus ambiguus (NA) and the hypoglossal nucleus (XII) in prenatal and adult specimens. This will assist in analysis and interpretation of results from the investigation into URT motor nucleus development.

Figure 4.1: Midsagittal section through the head and neck of an adult rat. Note the opposition of the epiglottis (E) with the soft palate (S). This creates an intra-narial larynx which anatomically defines distinct pathways for respiration and deglutition. As noted in chapter 1, this is considered a baseline mammalian URT morphology (from Reidenberg, 1988; used with permission of the author).

Figure 4.2: Histological section through the adult rat head and neck. As in the gross specimen (above), the epiglottis and soft-palate can be observed in opposition (at arrow). In addition, individual muscles of the tongue can be seen coursing longitudinally (along the superior surface) and vertically (within the body of the tongue). The genioglossus can also be seen radiating into the tongue from the mandible (from Reidenberg, 1988; used with permission of the author).

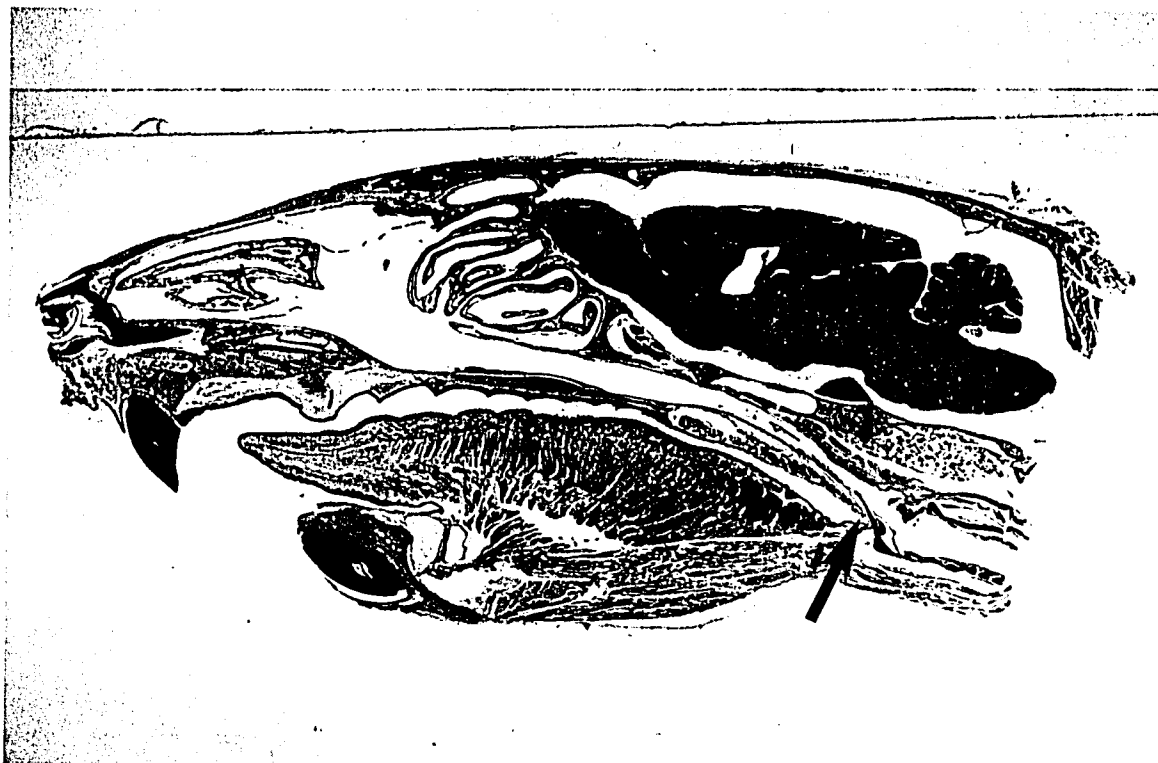


Figure 4.3: Histological section through an entire prenatal rat at 15 days of gestation (E15). The tongue is well developed and already a prominent feature of the oral cavity. The larynx (arrow), can be see developing with the epiglottis at the base of the tongue. At this stage, crown-rump length is approximately one centimeter.



Figure 4.4: An E15 rat and an isolated E15 central nervous system. The prominent "bulge" in the dorsal cranium can be seen to correspond to the cephalic flexure in the developing brain (large arrow). Caudal to this flexure are the pontine and cervical flexures (small arrows), between which is located the brainstem.

Figure 4.5: The central nervous systems of an E15, E17 and E19 rat (right-to-left). The unusual curvature of the E17 and E19 spinal cords does not represent the "*in situ*" morphology and is the result of post-dissection fixation. Note the dramatic increase in size of the brain and brainstem during this period. In addition, the nervous system appears to further differentiate during this time. For example, the cerebellum can be seen to be forming just dorsal to the rostral end of the brainstem.

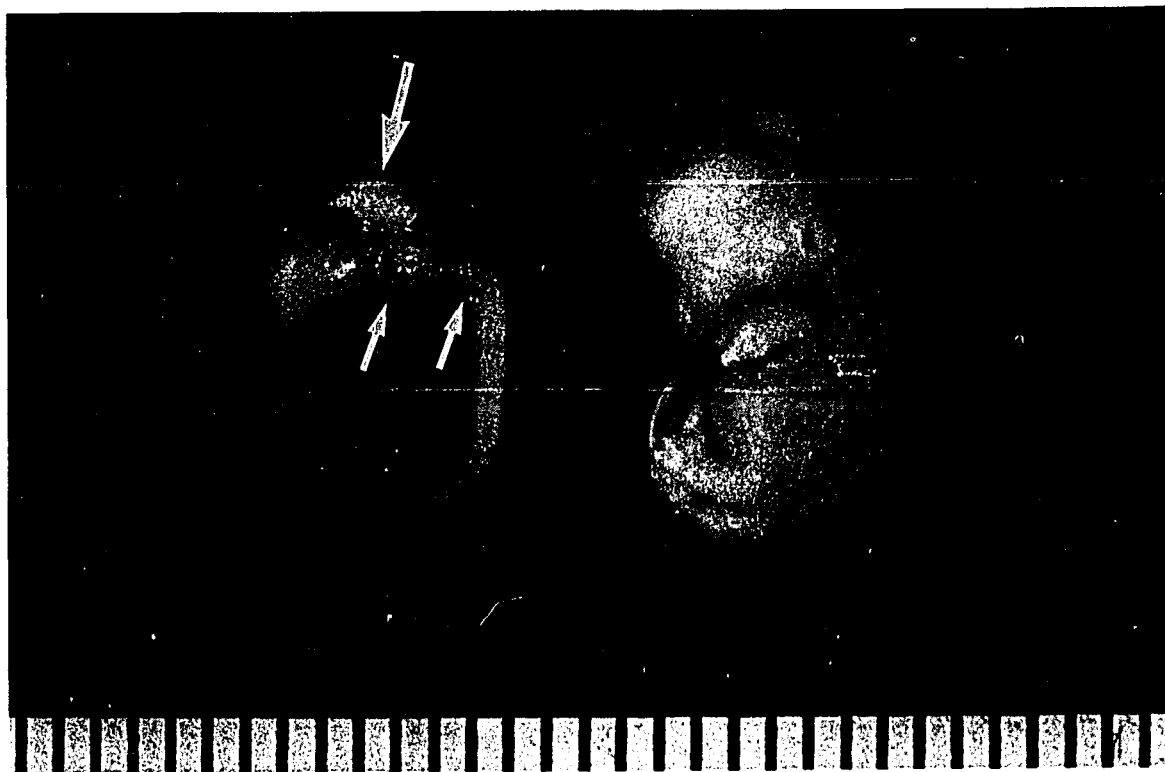


Figure 4.6: Transverse section through an adult rat brainstem at the level of rostral NA. The left and right NA can be seen as densely staining clusters of cells in the ventrolateral brainstem. The left NA is indicated by arrow. (cresylviolet, 16x)

Figure 4.7: Closer view of the above brainstem showing the adult NA on the left side. Motoneurons can be seen defining the compact formation of NA (see chapter 2). Distinct nucleoli are apparent in several cells. (cresylviolet, 80x)

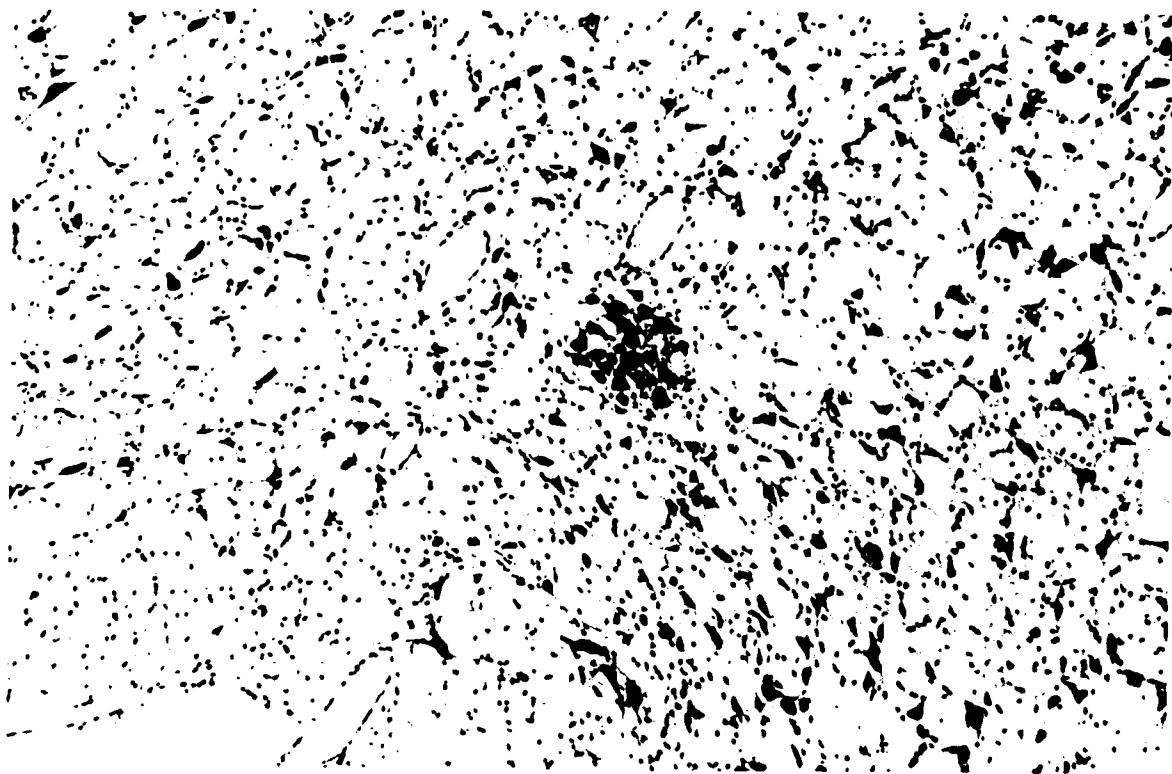


Figure 4.8: Transverse section through an adult rat brainstem at the level of XII. Note the left and right nuclei in the dorsomedial brainstem along the fourth ventricle. Left nucleus is indicated by arrow. This section is rostral to obex. Below obex XII lies more ventrally, just ventrolateral to the central canal. (cresylviolet, 16x)

Figure 4.9: Closer view of above brainstem. The left and right adult XII are well-defined and clearly separated in the midline (at large arrow). Immediately dorsolateral to each nucleus are small clusters of motoneurons of the dorsal motor nucleus of the vagus (shown on left side by small arrow). (cresylviolet, 40x).

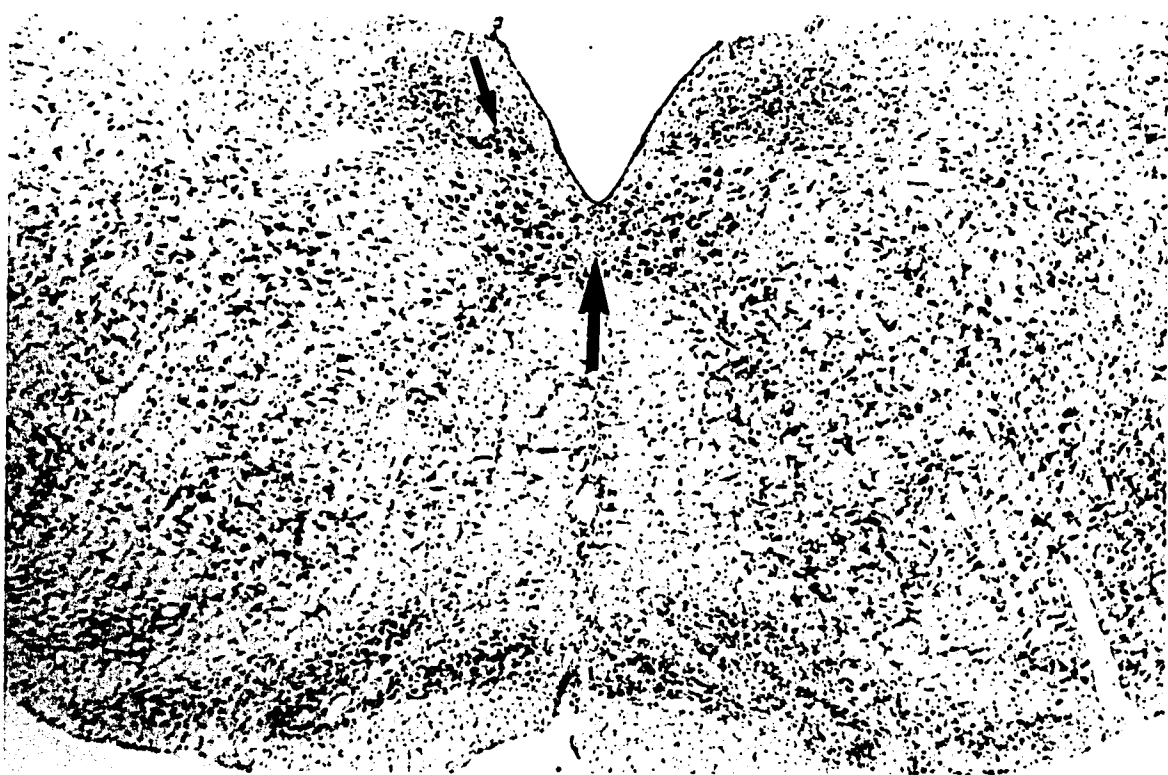


Figure 4.10: Transverse section through an E17 brainstem at the level of NA. This age demonstrates the general morphology of prenatal brainstems. Distinct clusters of cells defining NA can be seen in the ventrolateral brainstem on the left and right sides. Arrow indicates the left NA. Dorsal to the brainstem is the developing cerebellum. Note the differences in morphology and size between this specimen and the adult brainstem (figure 4.6). (hematoxylin and eosin, 40x)

Figure 4.11: Closer view of the above E17 brainstem on the left side. Motoneurons can be seen to define the compact formation of NA (arrow). Distinct nucleoli are apparent within these cells. (H&E, 125x)

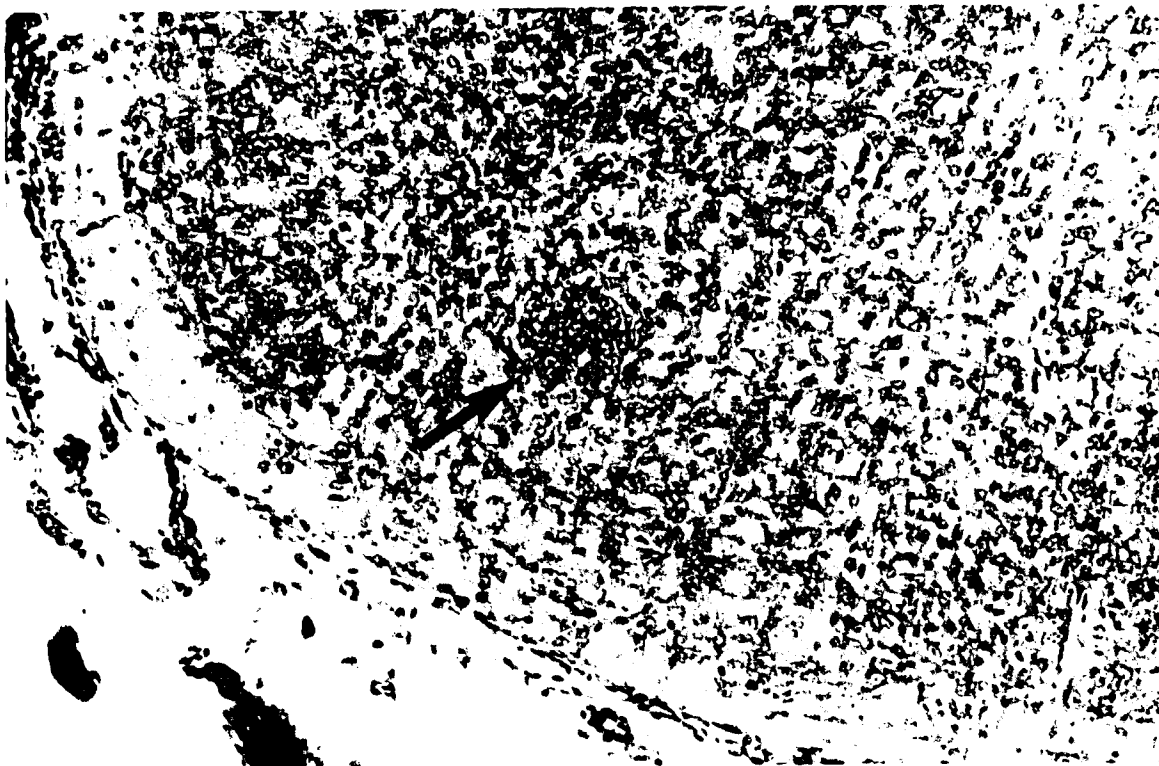
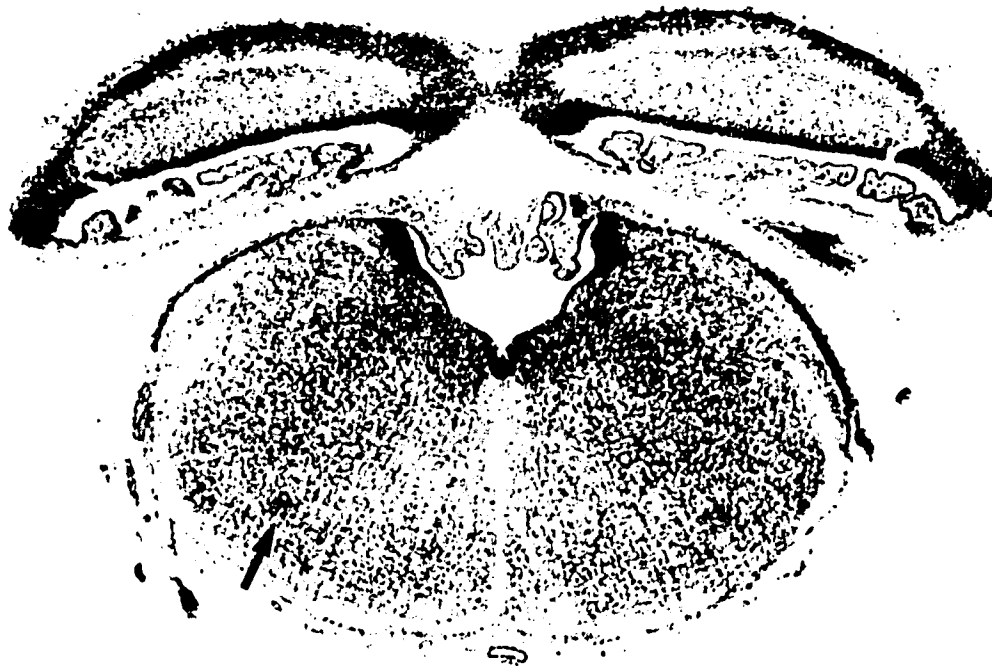


Figure 4.12: Transverse section through an E19 brainstem at the level of XII. As in the adult, this section was taken rostral to obex to demonstrate the nucleus along the fourth ventricle. Arrow indicates left XII. The nucleus is defined by small, darkly staining cells on the periphery (see below). (H&E, 40x)

Figure 4.13: Closer view of the above E19 brainstem showing the left and right XII (left indicated by arrow). The small cells defining the periphery of the nucleus can be seen (e.g., small arrow). In addition, motoneurons within the nucleus are apparent. (H&E, 80x)



Chapter 5

Methods For Investigating Motoneuron Cell Death

This chapter and the following five chapters will present the results of investigations into the development of the URT motor nuclei: the nucleus ambiguus and the hypoglossal nucleus. Due to the differences in methods of tissue processing and numbers of animals, as well as different sets of animals, the chapters have been separated into two groups. The first group (chapters 5, 6, and 7) will report experiments exploring and documenting the occurrence of motoneuron cell death (MCD). This chapter, chapter 5, will detail the materials and methods followed by result chapters on the nucleus ambiguus (chapter 6) and the hypoglossal nucleus (chapter 7). Chapters 8, 9, and 10 will report experiments investigating the formation of NA and XII and the development of GABAergic innervation within these nuclei. Materials and methods will be given (chapter 8), followed by results in the nucleus ambiguus (chapter 9) and hypoglossal nucleus (chapter 10). These last three chapters, although investigating the earliest developmental event studied, will be presented after the results of the investigation into MCD. This is because studies of nucleus formation and GABAergic innervation were initiated subsequent to the investigation of MCD with the specific aim of verifying the specificity of the motoneuron counts. Additionally, many of the methods used in the nucleus formation and GABAergic innervation studies were developed and refined in the earlier MCD studies.

5.1 Introduction

Motoneuron cell death (MCD), by definition, represents a decrease in the *number* of neurons in a particular motor pool. As such, the investigation of naturally occurring cell death requires quantitative assessment of the neuronal population. This allows objective statistical analysis of the data to demonstrate the patterns of cell loss in the neural region. Although indirect numerical measures such as weight of the tissue have been used (Detwiler, 1920), the majority of studies have sought to determine directly the number of neurons by counting cells (see review Oppenheim, 1991). There are many methodologies by which to count cells (see review Konigsmark, 1970), including recent attempts at computer automation (Greer et.al., 1983; Rauch et.al., 1990). However, most quantitative studies, including those on MCD, have relied upon direct microscopic observation of neurons or neuron-specific morphologies in serially sectioned tissue (Chu-Wang and Oppenheim, 1978b; Laing, 1982; Arens and Straznicky, 1986; Okada et.al., 1989). For reasons presented in the following sections, the investigation of MCD in URT brainstem motor nuclei also employed microscopic assessment of cells in serially sectioned material.

The counting of neurons in serially sectioned material requires the development of two specific protocols. The first addresses the histological processing of the tissue for microscopic evaluation. This entails fixation of the specimen, dissection of the organ under study, and sectioning and staining of the material. The second focuses on issues related to quantitative evaluation of cell populations. This employs mathematical arguments to evaluate sample size, sampling rate, errors of randomness, and the use of different counting profiles (i.e., what to count). The development of both protocols relied on preliminary investigations to determine the efficacy of histological procedures and to provide a mathematical description of the cell populations under study. The following sections will describe the pilot studies used to develop the final histological

and counting protocols for the investigation of MCD in the nucleus ambiguus and hypoglossal nucleus.

5.2 Pilot Studies: Histological Parameters and Techniques

Though the methods employed for this phase of the study are based upon traditional neuroanatomical and histological techniques, the different requirements of prenatal, newborn, and adult tissue necessitated the development of three separate protocols with their own distinctive parameters. The following sections will review the preliminary studies that helped to establish the final protocols for assessing MCD. Some of the work was concerned with the development of skills necessary to acquire the specimens and become familiar with the anatomy of the region. Additional work was experimental in nature and consisted of several different protocols directed at processing the tissues for cell counting. Other studies employed statistical methods to establish the parameters of cell counting such as sample size and sampling rate.

5.2.1 *Microdissection*

Microdissection and microsurgery must be learned and practiced in order to develop proper technique. Its importance to the surgeon has prompted the production of specific instructional material, including many books and video tapes, directed at teaching microsurgical methods (Acland, 1981; Berezniak, 1991). Furthermore, its importance in this study should not be overlooked, nor trivialized, as it was essential to the procurement of usable prenatal brainstem specimens for all facets of this thesis. Presented here are the main tenets of microdissection and the instruments and approach used in this study to remove prenatal rat brainstems.

The most important step in microdissection is reducing the natural tremor found in all individuals. This was done most effectively by resting the hands comfortably upon the dissecting table. This simple tenet dictated many additional aspects of the dissecting

"theater". For example, an adjustable height chair was used to allow the positioning of the arms as near to parallel to the floor as possible. The specimen was positioned so that the hands rested on their medial borders with minimal repositioning during dissection. Often times large gauze pads (4x4) were used to elevate the hands to the specimen height. Additionally, to minimize excess movement, the instruments were controlled principally by the fingers and not the hands or arms. Particularly, the instruments were held as a pencil, i.e., rested upon the ring finger for stability and moved by the thumb, middle and index fingers.

Equipment also plays a major role in determining the quality of dissected material. A tungsten fiber optic light source with flexible heads that were positioned near the specimen but outside the field of vision and dissection were used. The dissecting microscope was binocular with adjustable levels of magnification. Pedal-driven zoom and focus are generally recommended for maintaining hand position during the dissection (Acland, 1981). The majority of work in this study, however, was done on a manual zoom and focus dissecting microscope that required frequent adjustment and repositioning of the hands. The instruments most often used in this study were iris scissors, two number 5 jewelers forceps, a small Beaver blade, and a number 11 scalpel blade.

The removal of prenatal rat brainstems was designed after several preliminary dissections to understand the anatomy of the region. Landmarks in the dissection of prenatal rat brainstems included the developing eye, the otocyst, and the middle cerebral artery. A vertical incision was made with the Beaver blade anterior to the otocyst. This incision was made deep as it followed the course of the middle cerebral artery and was immediately posterior to the border of the developing telencephalon. A perpendicular incision was extended rostrally just superior to the developing eye. This corresponded to the skull base and was inferior to the developing CNS. These two incisions enable the "peeling back" of skin and cartilage over the developing forebrain. From the origin of

the initial incisions, a posterior incision inferior to the otocyst was made. Skin was then removed over the dorsal cranium. The above steps were repeated on the other side of the rat to join the vertical incisions at lambda.

At this point the developing forebrain and part of the midbrain were exposed. Using both forceps the occipital cartilage was separated along a midsagittal plane by pulling laterally. This exposed the developing cerebellum, fourth ventricle, and brainstem. Using the Beaver blade, skin was incised and removed over the lower medulla and upper spinal cord. The cervical vertebrae were then cut through their laminae on both sides to expose the entire brainstem and upper spinal cord. Using forceps and iris scissors, the brain was elevated off the skull base from rostral to caudal and the exposed cranial nerves cut. Specifically, the optic, trigeminal, vestibulocochlear, facial and glossopharyngeal-vagus nerves were cut in order. All other nerves were easily torn by elevating the CNS. A final transverse cut across the spinal cord completely isolated the brain and brainstem and allowed its removal for further processing.

5.2.2 Fixation Methods

Postmortem tissue used for histological studies must be fixed, that is, preserved in such a manner that autolysis and the loss of cytoarchitecture is prevented. The choice of fixative was critical to this study because of the need to identify specific cellular structures in the brainstems of many animals at various ages. Thus, the fixative must have ensured reproducibility in the preservation and appearance of motoneurons in all organisms studied as well as being compatible with the chosen staining protocols (Konigsmark, 1970).

A review of the MCD literature showed a number of different fixatives in use such as 4% paraformaldehyde, 4% paraformaldehyde with varying percentages of glutaraldehyde, glutaraldehyde alone, Carnoy's fixative, Bouin's fixative, and additional unnamed fixatives (e.g., Hughes, 1961; Pollack, 1969; Rogers and Cowan, 1973; Sohal,

1976; Chu-Wang and Oppenheim, 1978a; Oppenheim, 1981; Lance-Jones, 1982; Ashwell and Watson, 1983; Harris and McCaig, 1984; Comans et.al., 1987; Sengelaub and Arnold, 1989; Oppenheim et.al., 1989; Sohal et.al., 1990). General tissue fixation is most commonly done with 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS), commonly called formalin, or correctly, 10% formalin. As this solution had been used in studies of MCD, initial studies employed this solution for the fixation of rat brainstems.

Pilot Studies

The pilot studies were carried out in adult rats. This allowed the learning of proper transcardial perfusion technique in addition to testing fixatives. The protocol followed here for transcardial perfusion has been fully described by Friedrich and Mugnaini (1981). Adult rats were anesthetized with ketamine (0.0006 cc/g, i.p.) and rompun (0.0006 cc/g, i.m.). The inferior surface of the diaphragm was exposed via abdominal incision. The diaphragm was opened, the ribs cut and reflected, and the descending aorta clamped. The right atrium was incised and a cannula introduced into the ascending aorta through an incision in the left ventricle. The vascular system was flushed with 0.1M PBS for 1 minute followed by the fixative for 30 minutes. All perfusions were performed with a perfusion pump allowing high flow rates and pressure. After removal of the brainstems they were immersion fixed for one week and sectioned and stained as described below. The resultant brainstem sections were well stained and clearly showed NA and XII. Under high power the motoneurons in these nuclei appeared well preserved, densely stained, and exhibited distinct nucleoli. The fixation of neonatal material (P1) was also carried out with 4% paraformaldehyde. This was due to the ability to perfuse directly the organism and thus adequately penetrate the brainstem with

fixative. When the investigation of prenatal tissue was started, a similar protocol was employed.

For the investigation of prenatal brainstems, the mother was transcardially perfused with 4% paraformaldehyde and the offspring removed from the uterine sacs for subsequent immersion fixation. After approximately one week in fixative, the brainstems were dissected out, sectioned, and stained. The quality was extremely poor and, although NA and XII were able to be located, they were indistinct and cellular morphology was indistinct. The protocol was modified so as to open the cranium and expose the brainstems to fixative during immersion fixation but the results were no better. These results indicated the need for a more rapidly penetrating fixative.

Carnoy's fixative is a rapidly penetrating solution of alcohol, chloroform, and acetic acid in a 6:3:1 ratio, respectively. Many studies on MCD in the chick embryo have used immersion in Carnoy's fixative (e.g., Hollyday and Hamburger, 1976; Chu-Wang and Oppenheim, 1978a; Hamburger et.al., 1981; Arens and Straznicky, 1986). It was not used initially because of difficulties in adapting an avian protocol for a mammalian study. For example, in avian studies, the tissue undergoes little "lag" time between death and contact with the fixative as the embryo is simply removed from the egg and placed in fixative. The equivalent in rats would be a surgical approach to both uterine horns and the individual removal of prenatal rats for Carnoy's immersion. This, however, would expose the prenatal rat to many stresses (e.g., long periods of anesthesia, and hypoxia and hypovolemia associated with surgery). The possibility that these stresses may have caused some neuronal death would have prevented accurate assessment of motoneuron populations. Thus, a protocol was designed in which the pregnant rat was directly and rapidly perfused with Carnoy's solution. This was intended to minimize the time between death and fixation in prenatal rats.

The first attempt at using Carnoy's solution found that the fixative lysed blood cells and resulted in the rapid formation of clots preventing adequate perfusion. Additionally,

Carnoy's was not perfused, but rather, *diffused* across the placental barrier into the fetal vasculature. Thus, introducing any amount of fixative to the uterine arterial system was as effective as a prolonged perfusion. Therefore, the perfusion protocol was modified as follows: 1) An initial flush with 0.1M PBS to remove blood and prevent clotting. This was for no more than 30 seconds and carried out at high pressure and flow rate. 2) The flush was immediately followed by perfusion with Carnoy's at high rate and pressure until it was seen to blanch the uterine walls. This typically took no more than five minutes and ensured that all the offspring were exposed to some Carnoy's fixative immediately postmortem.

After perfusion, the uterus was removed, the walls were cut between each sac and the entire uterus immersed in Carnoy's solution during the processing of individual prenatal rats. Each uterine sac was taken from the fix, the uterine wall, amniotic and chorionic sacs opened and the fetus removed. The rat was weighed and in most specimens crown-rump lengths were taken. Some of the initial specimens were only weighed as characterization by crown-rump lengths was suggested and adopted later in the investigation. The occipital and parietal bones (cartilage at this stage) were removed to expose the brain and brainstem to fixative and the entire fetus immersed in fix for approximately three hours. Six offspring from each litter, selected at random, were processed in this manner for MCD assessment. The time between dissecting the first and last fetuses was never more than 15 minutes ensuring a minimum of architectural loss in the latter.

Initial studies were carried out with focus on the hypoglossal nucleus which was well preserved and stained clearly. When attention shifted to the nucleus ambiguus it was seen that in many specimens the ventral portion of the brainstem had not fixed properly. It was found that the practice of opening the cranium allowed diffusion of the fixative into the dorsal brainstem but in three hours time it had not sufficiently penetrated through the ventral portions. Thus, all subsequent rats were immersion fixed for 30 minutes, the

entire brain and brainstem dissected out, and then re-immersed in fixative for an additional 2.5 hours. This exposed the ventral surface to fixative allowing diffusion to the nucleus ambiguus. The protocol for dissection was changed rather than extending immersion time after a pilot study showed tissue damage after longer immersions.

These pilot studies established the use of Carnoy's fixative in prenatal animals and 4% paraformaldehyde in postnatal specimens. The use of different fixatives pre- and postnatally did not create artifactual differences in motoneuron counts. This was due to the compatibility of both fixatives with the respective staining techniques allowing the consistent demonstration of brainstem motoneurons. The initial problems with fixation in the ventral brainstem of prenatal rats required the use of additional specimens for the investigation of MCD in NA. Thus, many rats in this study were unique to the investigation of NA.

5.2.3 Tissue Sections

The determination of cell numbers in NA and XII relied upon serially sectioned tissue. As described in section 5.3, serial sections were taken through the entire nucleus, cells counted in each section, totaled, and the raw counts multiplied by the sampling rate (for methodological review see Konigsmark, 1970). The reliance on regularly repeated sections of the same thickness made the method by which sections were taken critical to the assessment of cell numbers. There are many different methods for sectioning material and several were tried in an effort to acquire consistently sections of sufficient quality for motoneuron assessment.

Pilot Studies

Adult rat brainstems from the aforementioned pilot study were used to test sectioning methodology as well as determine optimum section thicknesses. Many studies that have

counted cells in the adult brain sectioned tissue at 20 μ m. Sections of this thickness are often obtained by cutting frozen tissue on a sliding microtome or in a cryostat. Both these methods were tried but neither produced the quality nor reproducibility necessary for this study. Specifically, consistent periodic serial sections were often uncollectable due to cutting damage and many sections collected often exhibited tattering and freezing artifact.

One of the most common methods for producing quality sections in a consistent and reliable manner is the use of paraffin. Paraffin is a wax-like substance in which tissue can be embedded and then sectioned on a table-top rotary microtome. Although more reliable than frozen sections, preparing the tissue for embedding and the embedding itself is a more lengthy process. Adult rats were perfused and fixed as described above. The brainstems were dehydrated through graded alcohols, cleared in xylene, and placed in melted paraffin overnight at 55-57°C. This last step was done in a vacuum oven to facilitate the penetration of paraffin into the tissue. Finally, the tissue was oriented in melted paraffin and then cooled on ice to form a semi-hard block for sectioning.

The initial paraffin sections fell apart while being cut. This was determined to be due to insufficient dehydration and clearing of the tissue prior to embedding. The protocol was adjusted so that adult brainstems were dehydrated for at least two hours in each alcohol and cleared in xylene overnight (preliminary studies in prenatal brainstems found that clearing in xylene could be accomplished in as little as two hours). After clearing, sections of adult brainstems were cut at 20 μ m. As described in section 5.2.4, motoneurons in the adult NA stain particularly densely for Nissl. At 20 μ m these motoneurons sometimes obscured each other and prevented accurate counting. That is, sections cut at this thickness required excessive adjusting of focal planes during counting and nucleoli were often observed only by focusing through other cells. It was found that 10 μ m produced good serial sections that stained well and allowed accurate counting of

NA and XII. Subsequent studies showed that this section thickness also worked well for newborn brainstems.

Sectioning of prenatal tissue was initially prevented by difficulty in orienting the brainstems in the wax blocks. Adult brainstems are large and were easy to manipulate so that NA and XII could be embedded perpendicular to the knife. Prenatal brainstems, however, are only several millimeters across and were difficult to orient in the solidifying wax. Thus, an adjustment was made in the dissection protocol to preserve the forebrain. This allowed a transverse section to be made across the cortex which provided a suitable "base" upon which to "stand" the brainstem during embedding. This oriented NA and XII perpendicular to the knife and established a sectioning direction from rostral to caudal.

Prenatal tissue was initially sectioned at 10 μ m like the adult and newborn material. The small size of the immature neuroblasts created many focal planes and overlapping cells. Section thickness was thus reduced to 5 μ m, however, there were two problems with this thickness: 1) NA and XII were difficult to discern because of the few number of motoneurons in each section representing them, and 2) consistent serial sections were difficult to obtain with an acceptable degree of reliability. All prenatal tissue was thus cut at 8 μ m at which NA and XII were clearly defined, and virtually every section exhibited good quality.

After sections were cut they were prepared for staining by deparaffinizing the tissue through rehydration from xylene to water. During this process many sections fell off the slides preventing staining. Many different media are used to coat slides so that they bind to the sections without disturbing the staining characteristics. The most frequently used solution is chrome alum, a 1% solution of gelatin and chromium aluminum sulfate. This solution was tried but during staining many sections continued to fall off the slides. As such, an alternative protocol was found and adapted from the Armed Forces Institute of Pathology Histology Manual (Luna, 1968). It consisted of adding approximately 200ml

of 5% gelatin to the water bath used for collecting the paraffin sections. After filling a slide with sections it was placed in a 57°C oven for ten minutes to soften the paraffin and adhere the section. Once this protocol was employed virtually no sections were lost during the staining of slides.

5.2.4 Tissue Stain

The most common method for the demonstration of neurons, and in particular motoneurons, is to stain for Nissl (Konigsmark, 1970). Nissl substance is the name given to the dense clumping ribosomal RNA on the endoplasmic reticulum as seen through the light microscope. This RNA is strongly basophilic and stains intensely with any of the general basic histological dyes. Nissl stains, by their basic nature, also clearly stain the cell nucleus and nucleolus. Additionally, these stains generate very little background staining as the majority of neuropil is acidophilic and thus remains unstained. For these reasons, Nissl stains are frequently used to assess cell numbers in various motor nuclei and sympathetic ganglia (Konigsmark, 1970). One of the most commonly used of these dyes is cresylviolet.

Pilot Studies

Adult rat brainstems used in the previous pilot study on fixatives were sectioned and stained with cresylviolet. The motoneurons stained densely and distinctly and the nucleus ambiguus and hypoglossal nucleus were clearly defined. Furthermore, motoneurons in NA were found to stain particularly densely for Nissl. Nucleoli were distinct, round, and prominently situated in clear nuclei.

Prenatal tissue fixed with Carnoy's was stained for Nissl. Neither NA nor XII were readily apparent as the staining was extremely light and cells not clearly defined. Additionally, the nucleus and nucleolus were not readily apparent and not suitably

demonstrated to perform cell counts. A review of the literature showed several studies which had not used Nissl stains, but rather combinations of basic and acidic dyes (e.g., Pollack, 1969; Chu-Wang and Oppenheim, 1978; Laing, 1982; Lance-Jones, 1982; Ashwell and Watson, 1983; Arens and Straznicky, 1986). Thus, hematoxylin and eosin was tried on prenatal specimens in suitably fixed tissue. Examination of the brainstem showed that NA and XII were distinct and obvious. The cells showed prominent nucleoli in a clear nucleus on a contrasting generally uniform background. In later prenatal tissue, the cell cytoplasm stained more densely and provided an even more distinct appearance to NA and XII.

The newborn brainstem had elements of both the prenatal and adult brainstems. For example, while the whole brainstem retained an immature morphology, the individual motoneurons resembled classic motoneurons rather than early neuroblasts. Thus staining was tried with H&E and with cresylviolet. The use of H&E provided too much background and made cell counting difficult. Cresylviolet staining was found to be very light, however, would have been appropriate for counting if the cells were darker. As such, the protocol for cresylviolet staining was reassessed. Specifically, one of the critical steps in Nissl staining is called differentiation. It essentially lightens the stain by immersion in acidified alcohol for an experimentally determined time (usually 30-60 seconds). Due to the aforementioned density of staining seen in the nucleus ambiguus the differentiation time was set at about 45 seconds and was similarly applied to the newborn tissue. Revising the protocol by shortening this step to about 25 seconds provided adequate staining of newborn tissue for assessment of cell numbers.

5.3 Pilot Studies: Cell Counting and Statistical Methods

The above tissue processing techniques were used because of their ability to provide consistent quality sections for the assessment of motoneuron numbers. Other aspects of

cell counting, however, were devised on an objective basis, that is, by statistical analysis of preliminary studies. These included: the sampling rate, or periodicity of serial sections; the sample size; and the evaluation of correction factors due to splitting and errors of randomness. The following sections will present the results of preliminary studies and statistical methods used to devise a final protocol for the assessment of motoneuron cell death.

5.3.1 Cell Counting Methodology

Once the methodologies for histological processing of rat brainstems were established, the resultant sections through URT motor nuclei were able to be examined for the occurrence of MCD. In order to demonstrate and characterize MCD, a quantitative measure reflective of a change in cell numbers needed to be taken at various developmental stages. As this is a study of cell death, initial inclination may be toward determining the number of dying cells at each age. Counting of degenerating cells, however, poses several problems. First, many of the degenerative changes are difficult to discern under the light microscope (Walker et.al., 1988) implying that significant numbers of dying cells would be uncounted. Second, because apoptotic cells undergo rapid dissolution and removal from the intercellular space (Searle, 1982; Kerr and Harmon, 1991), counts would have to be made in many precisely timed pregnancies only hours apart in gestation in order to observe all dying cells. Third, degenerating cells exhibit many morphologies (Wyllie, 1981; Walker et.al., 1988) preventing the establishment of a consistent criteria for inclusion in the cell count. Additionally, the various sizes and morphologies of degenerating particles would prevent the use of standard correction factors for splitting of the counted profile (see below). Thus, rather than count dying cells, this study focused on counting apparently healthy neuroblasts and motoneurons in order to assess developmental changes in URT motor nuclei. The

amount of cell death, therefore, was determined by changes in the number of *surviving* motoneurons.

A protocol for counting cells must establish an objective criteria for identifying the cell. Although some studies have counted the cell body, most quantitative studies count nuclei or nucleoli (Konigsmark, 1970). These subcellular structures are readily identifiable, exhibit well-defined and characterized morphologies, and are typically found once per cell. Furthermore, nuclei and nucleoli are significantly smaller than the cell body and thus errors in cell counts due to splitting would be reduced (for a constant section thickness) (Abercrombie, 1946). Splitting occurs when the unit counted lies near the surface of the section and is cut by the knife so that it appears in contiguous sections. In such a case it would be counted twice and the reported number of cells would be higher than the actual population. As will be shown mathematically below, the error due to splitting is proportional to the size of the counted unit relative to section thickness. As such, an effective means of reducing counting error would be to count the smallest possible profile, the nucleolus. As such, the present study on MCD relied on counts of neuroblasts and motoneurons exhibiting distinct nucleoli in NA an XII of the rat brainstem.

Many formulas have been devised and revised in order to correct for the splitting of nucleoli, the most common being that of Abercrombie (1946) and the revision by Konigsmark (1970). Recently, techniques such as optical dissection and three-dimensional-counting have sought to refine the correction process (Coggeshall and Chung, 1984; Coggeshall et.al., 1984; Williams and Rakic, 1988; Coggeshall et.al., 1990; Moller et.al., 1990; Nurcombe et.al., 1991). However, classic studies on MCD pre-date these refined counting techniques and thus, perhaps due to historical precedent, current studies often rely on the Abercrombie and Konigsmark formulas. Furthermore, a substantial number of MCD studies do not correct their counts for split nucleoli. The decision of whether to apply correction factors or not is based upon the nature of the

study, the methods used, and whether such factors have significant effects on the final results.

This study chose not to employ correction factors. The first reason for not using correction factors related to the use of paraffin embedded material. Paraffin is a soft embedding media and some researchers consider that nucleoli are rarely bisected in such substances. Rather, it has been suggested that nucleoli are pushed by the knife into one section or the other and thus are rarely, if ever, split (Jones, 1937; Tomasch and Malpass; 1958; Cammermeyer, 1967). Experiments direct at this issue, however, show differing results. Some researchers have observed no nucleolar splitting (Tomasch and Malpass; 1958) or very infrequent splitting (Jones, 1937; Cammermeyer, 1967), while others have seen considerable splitting (Coggeshall and Chung, 1984). These conflicting results may represent differences in histological techniques or hardness of the paraffin. Jones (1937) controlled for differences in technique by cutting serial sections from the same block of tissue at different thicknesses. If splitting occurred, one would expect there to be more splitting in the thinner sections and thus corresponding differences in nucleolar counts from those taken in thicker sections. Jones, however, found no appreciable difference in nucleolar counts regardless of section thickness thus arguing against the use of correction factors.

Aside from the use of paraffin, correction factors were not used in this study after their calculation showed they would have no effect on the experimental results. Figure 5.1 shows the calculations based on the Konigsmark (1970) formula in which the ratio of nucleoli in a section (N_i) to the number of nucleoli counted in that section (n_i) is the ratio of the section thickness (t) to the section thickness plus the size of the nucleolus corrected for microscopic resolution ($2a$). The calculations used observed nucleolar diameters at prenatal and adult stages of $2.375\mu\text{m}$ and $3.325\mu\text{m}$ and their respective section thicknesses of $8\mu\text{m}$ and $10\mu\text{m}$ (see section 5.2.3). The correction factor (as per figure 5.1) for prenatal specimens was 0.787 and for adult specimens 0.760. The similarity at

Figure 5.1

Calculation of Correction Factors for Split Nucleoli

$$\frac{N_i}{n_i} = \frac{t}{t+2a}$$

N_i = number of nucleoli in section

n_i = number of nucleoli counted in section

t = thickness of section

where $a = \sqrt{r^2 - (k/2)^2}$

a = corrected nucleolar size

r = radius of nucleolus k = smallest visible particle ($1\mu\text{m}$)

Prenatal Brainstems

$$r = 1.19\mu\text{m} \quad a = \sqrt{(1.19)^2 - (1/2)^2} = 1.080$$

$$t = 8\mu\text{m}$$

$$\frac{N_i}{n_i} = \frac{8}{8+2(1.080)} = 0.787$$

Adult Brainstems

$$r = 1.66\mu\text{m} \quad a = \sqrt{(1.66)^2 - (1/2)^2} = 1.583$$

$$t = 10\mu\text{m}$$

$$\frac{N_i}{n_i} = \frac{10}{10+2(1.583)} = 0.760$$

both stages established an essentially constant correction factor across the entire study. Thus, because MCD was assessed by comparing relative changes in cell number between ages, applying a constant correction factor had no effect on the results. For example, if 50% cell loss was observed between the earliest and latest ages, applying a constant correction factor would not alter the 50% relationship. Therefore, this study did not use correction factors for split nucleoli as splitting in paraffin embedded tissue appears to be insignificant and the use of different section thicknesses between prenatal and adult stages mathematically negates the effect of correction factors on the experimental results.

5.3.2 Preliminary Cell Counts

The preliminary studies into MCD were designed with three purposes: 1) to determine if MCD occurs in brainstem URT motor nuclei; 2) to determine the approximate gestational ages during which MCD occurs; and 3) to determine the number of motoneurons in NA and XII of mature and immature rats in order to establish a final counting protocol by statistical methods. With regard to the third concern, only two studies were found in which cells were counted in either nucleus (Lewis, 1971; Yu, 1988). These studies counted XII in adult rats and found approximately 3500 motoneurons. No studies were found that counted cells in the adult NA, or prenatally in either nucleus.

For the preliminary studies into MCD, brainstems of E17 and E19 rats were sectioned at 8 μ m, every third section stained with hematoxylin and eosin, and nucleoli counted in the left and right NA and XII. A sampling rate of every third section is a comparatively high rate (see review Konigsmark, 1970) and was considered to provide reasonable estimation of the number of cells in each nucleus. Adult brainstems were sectioned at 10 μ m and every fifth section stained with cresylviolet and nucleolar counts made in the left and right NA. The greater size of the adult brainstem allowed the use of a lower,

although also relatively high sampling rate. Motoneuron numbers for the adult XII were obtained from Lewis (1971).

Results of the pilot study are summarized in table 5.1 which indicates the animal, the nucleolar counts and the estimated total cells for NA and XII. The animal codes (e.g., "E17-A1") reflect their ages (e.g., "E17"), their litter (e.g., "A"), and a specimen number (e.g., "1"). The counts for each section, taken from rostral to caudal, were summed to provide the "nucleoli counted" which were then multiplied by the periodicity to determine the "estimated total cells". Data is shown for the left and right nucleus, and averages provided for each animal and age. Raw nucleolar counts for each section are shown in Appendix A.

5.3.3 Sampling Rate

As described above, motoneuron numbers were determined by counting cells in serial sections through NA and XII. The size of these nuclei and the large number of cells they contained precluded the counting of every section through the nucleus. Thus, a protocol was established by which sections were sampled at a regularly repeated rate and nucleoli counted in these sections. The number of nucleoli counted was then multiplied by the sampling rate, also called the periodicity, to provide an estimate of the total cells in the nucleus.

The estimation of motoneuron numbers by sampling carries with it inherent errors due to the random distribution of cells throughout the nucleus. Specifically, because total cell numbers were estimated by sampling a randomly distributed population, the accuracy of the estimate was limited by the standard deviation of the Poisson distribution: $\sigma = (np)^{1/2}$. In this equation, "n" is the number of nucleoli sampled and "p" is the probability of any nucleolus being included in the sampled set of nucleoli. In order to establish an appropriate sampling rate, i.e., one that would provide reasonable estimation of total cell numbers, preliminary counts were taken at a high sampling rate

Table 5.1
Preliminary Studies on Motoneuron Cell Death
Motoneuron Counts

Nucleus Ambiguus

	Nucleoli Counted				Estimated Total Cells		
	L	R	Average		L	R	Average
E17-A1	1032	1046	1039	⋮	3096	3138	3117
E17-A2	952	948	950	x 3	2856	2844	2850
Average:			<u>994.5</u>	⋮			<u>2983.5</u>
E19-A3	638	619	628.5	⋮	1914	1857	1885.5
E19-B1	679	654	666.5	x 3	2037	1962	1999.5
Average:			<u>647.5</u>	⋮			<u>1942.5</u>
A-1	313	299	306	⋮	1565	1495	1530
A-2	257	257	257	x 5	1285	1285	1285
Average:			<u>281.5</u>	⋮			<u>1407.5</u>

Hypoglossal Nucleus

	Nucleoli Counted				Estimated Total Cells		
	L	R	Average		L	R	Average
E17-A1	1584	1528	1556	⋮	4752	4584	4668
E17-A2	1465	1389	1427	x 3	4395	4167	4281
Average:			<u>1491.5</u>	⋮			<u>4474.5</u>
E19-A3	1228	1256	1242	⋮	3684	3768	3726
E19-B1	1241	1170	1205.5	x 3	3723	3510	3616.5
Average:			<u>1223.75</u>	⋮			<u>3671.25</u>
Adult	(calculated from Lewis et.al., 1971)			⋮	(Lewis et.al., 1971)		
Average:			<u>698.8</u>	x 5			<u>3494</u>

and the results of lower sampling rates extrapolated from this data. Subsequently, a final sampling rate was determined by selecting for the lowest acceptable deviation among the different rates thus ensuring good estimation of total cell numbers in both URT motor nuclei.

The pilot study was used to extrapolate the results of decreasing the sampling rate in prenatal specimens. This was done by re-sampling the raw data for each section, already sampled at every third, to determine the results of sampling every sixth, ninth, and twelfth sections. The raw data for these extrapolations are shown in Appendix A, and the results graphed in figures 5.2 and 5.3. Figure 5.2 shows the effects of lower sampling rates in NA at E17 and E19. The filled circles represent the estimated number of cells in the left and right nuclei, while the cross and error bars demonstrate the mean and standard deviation, respectively. It is apparent at both ages that as sampling rate is decreased (toward the right), the standard deviation is increased. Figure 5.3 shows the results of lower sampling rates in XII at E17 and E19. Although a similar trend is apparent, counts of every ninth section at E19 showed a very small standard deviation, however, the mean was elevated as compared to every third. Figure 5.4 graphically represents the effect of lower sampling rates on the standard deviations in NA and XII in both E17 and E19 brainstems. It is apparent that between sampling rates of every ninth and every twelfth sections, the standard deviation becomes extremely volatile. Furthermore, sampling at every ninth may be equally volatile as indicated by the unusual result in the hypoglossal nucleus at E19. Thus, a sampling rate of no lower than every sixth section should result in reliable estimations of total cell numbers in prenatal brainstem URT motor nuclei.

The above extrapolations helped to establish a minimum sampling rate at which the standard deviation is sufficiently small so as to produce reliable estimations of total cell numbers in a single population. The assessment of MCD, however, relies on comparisons of estimations between populations at different ages. As such, the standard

Figure 5.2
Estimated Total Cells in the Nucleus Ambiguus
The Effect of Different Sampling Rates

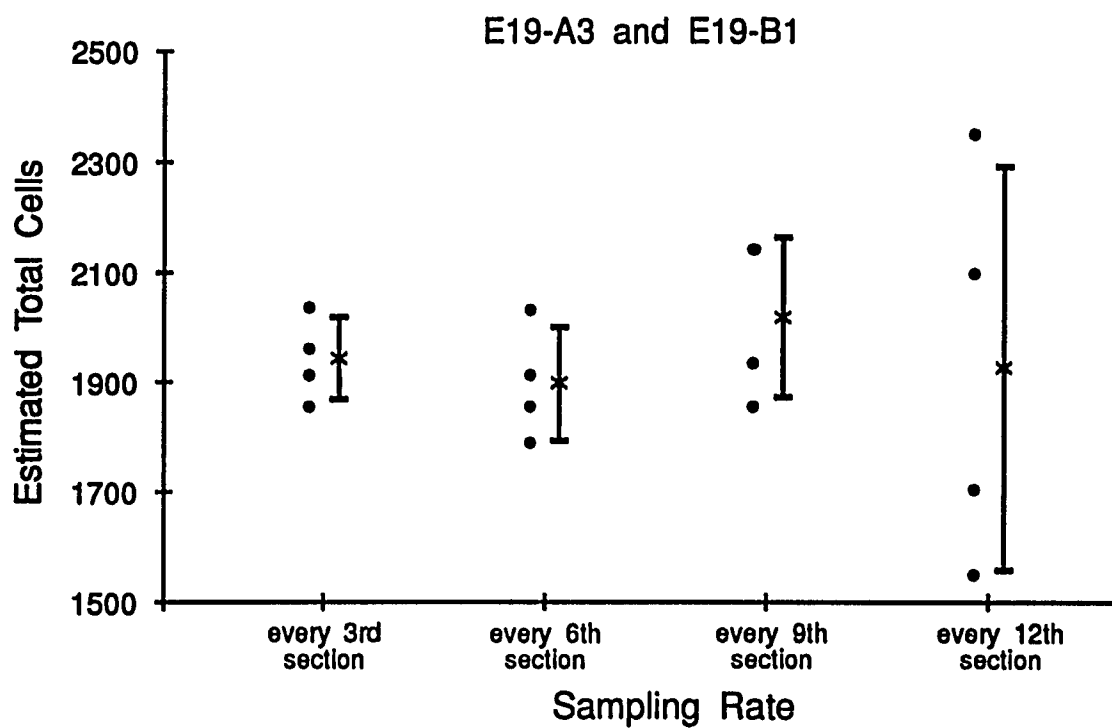
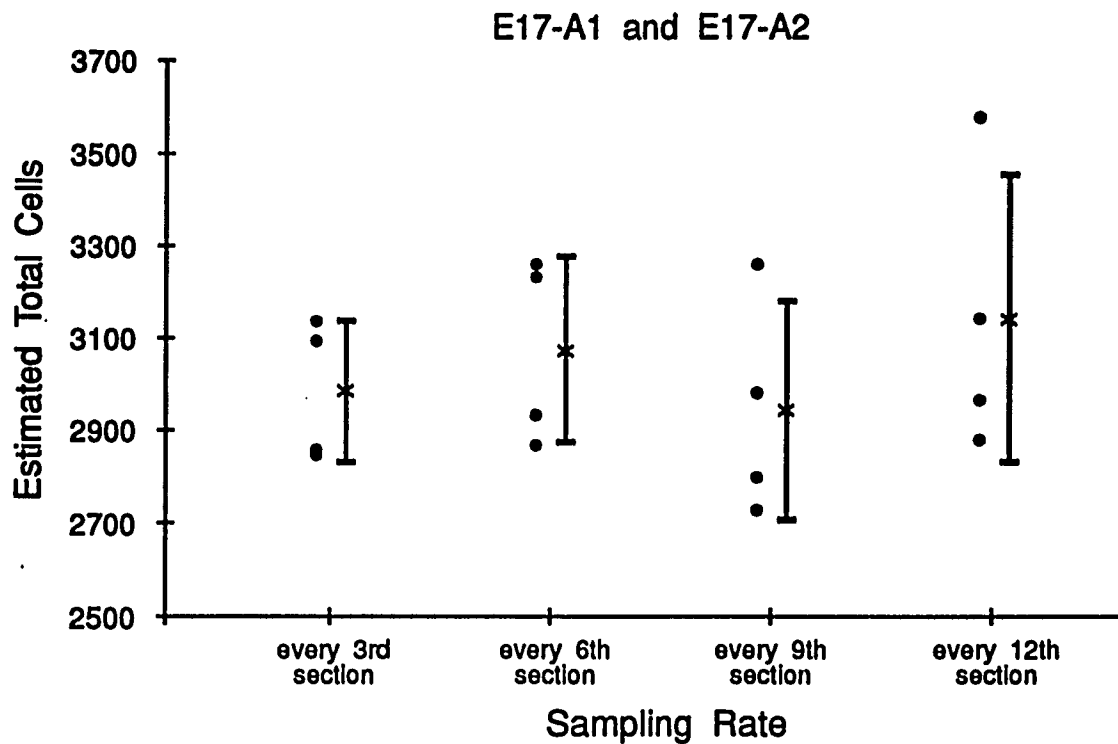


Figure 5.3
Estimated Total Cells in the Hypoglossal Nucleus
The Effect of Different Sampling Rates

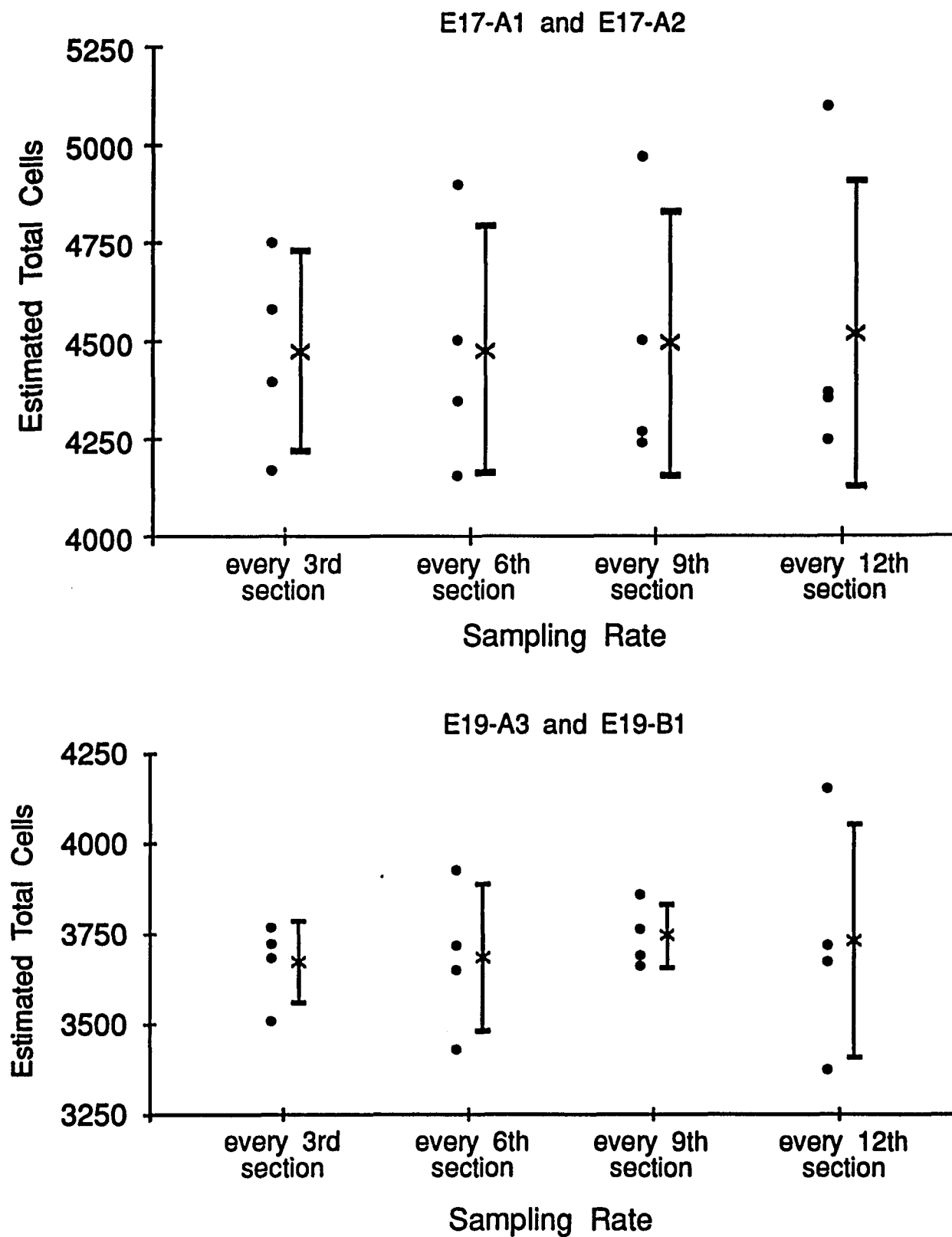
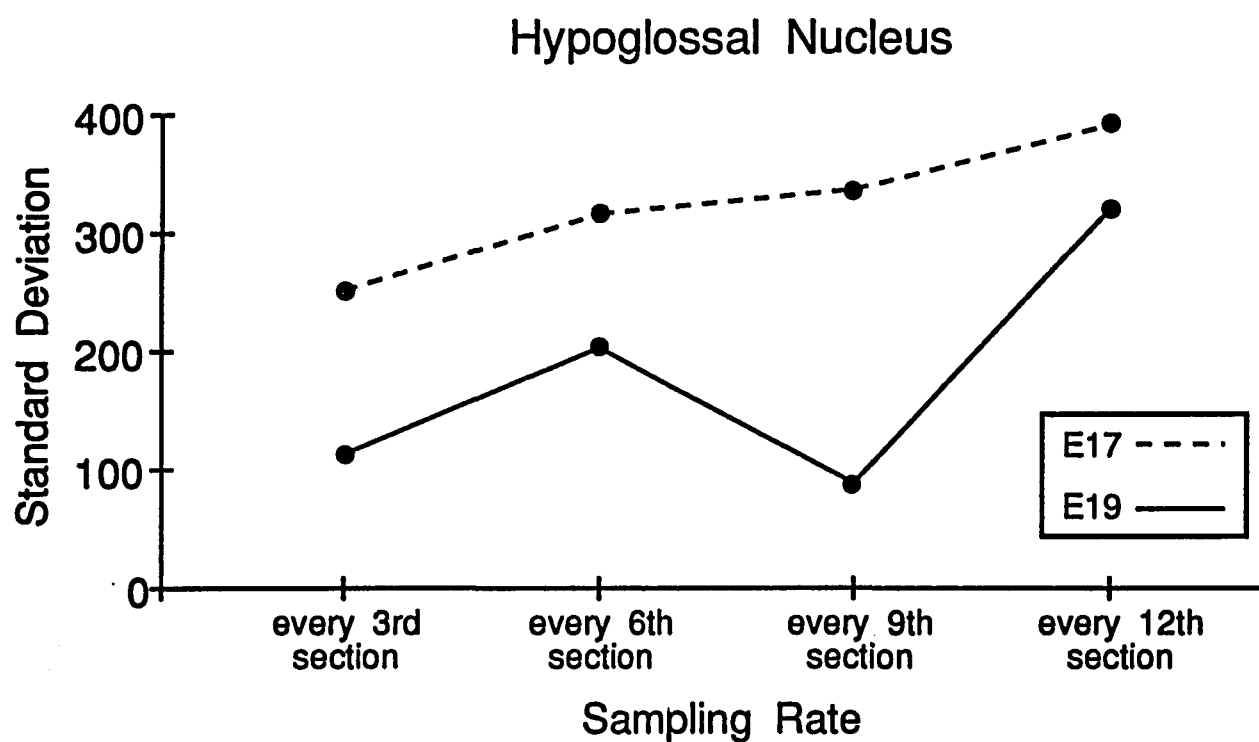
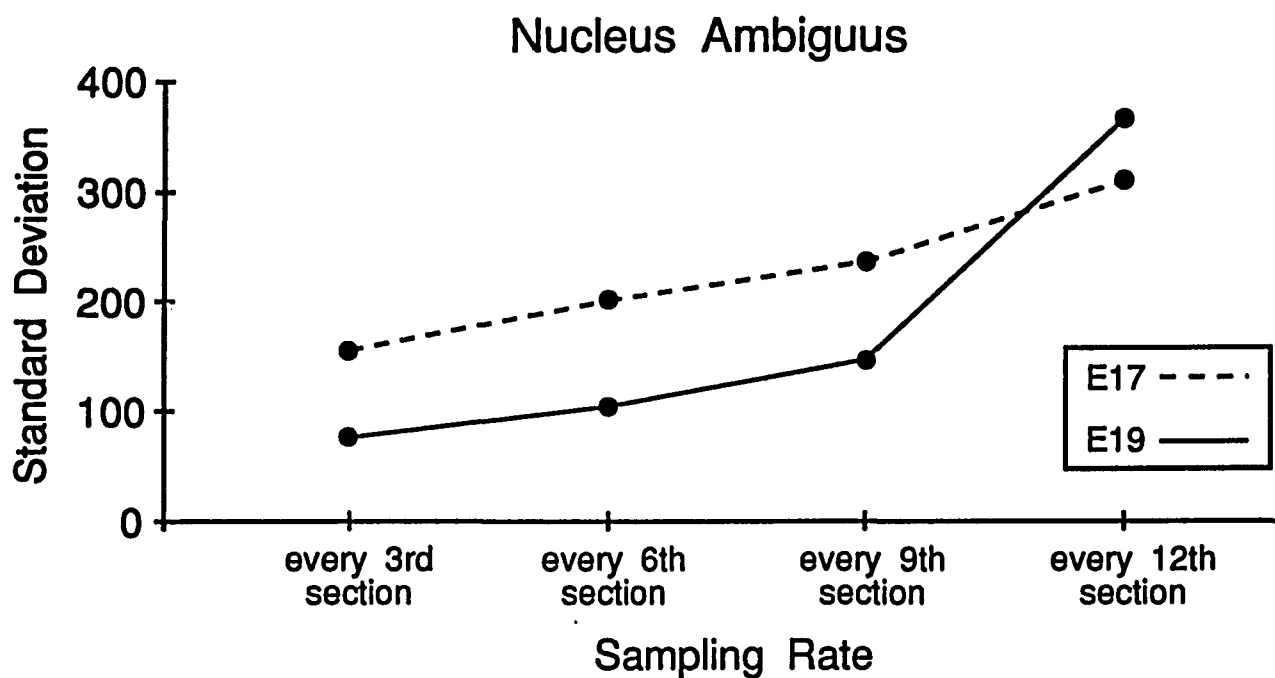


Figure 5.4
Standard Deviation of Estimated Total Cells
The Effect of Different Sampling Rates



deviations, in addition to being reasonably small, should be similar at each age such that errors of randomness are constant throughout the study. That is, similar errors in the estimation of total cells at each age would have no effect on the relative changes in cell numbers between ages, in the same way similar correction factors for split nucleoli had no effect on the changes in cell number (see section 5.3.1). In order to minimize such error, one must re-examine the standard deviation in randomly sampled populations.

As noted, the standard deviation for a random sample was dictated by the Poisson distribution as:

$$\sigma = (np)^{1/2}$$

where "n" is the number of nucleoli able to be sampled and "p" is the probability of any nucleolus being included in the sampled set. Thus, the variable "p", is directly related to the sampling rate as:

$$\frac{1}{\text{sample rate}}$$

Furthermore, because "n" is the number of nucleoli able to be sampled, it is equal to all the nucleoli in the nucleus and, as such, is equivalent to the estimated total cells, a fixed variable. Thus, in order to generate similar standard deviations at each investigated age, only "p" could be modified, which was accomplished by adjusting the sampling rates within the limits established above. However, because the sampling rates were relatively high, "p" became too large to describe the deviations by the Poisson distribution. Rather, the standard deviation had to be described by the binomial distribution as:

$$\sigma = (np(1-p))^{1/2}$$

Using this formula, standard deviations before and after modifications in the sampling rate were calculated at E17, E19, and adult stages for NA and XII. These results are shown in figure 5.5 which compares the standard deviation using the pilot study sampling rates with the standard deviation using proposed sampling rates. Note the similarity within each nucleus of standard deviations at E17, E19 and adult ages after changing the sampling rate, as compared to the standard deviations in the pilot studies. It was found that a prenatal sampling rate of every fifth section (i.e., a "p" of 0.20) and an adult sampling rate of every third section (i.e., a "p" of 0.33) were able to reduce the differences in standard deviations between ages. Furthermore, both these sampling rates, i.e., every third and every fifth, were higher than the every sixth rate derived above and would thus produce accurate estimations of total cell numbers in the URT motor nuclei.

In summary, prenatal and postnatal day one tissue was sampled at every fifth section and adult tissue sampled at every third section. These rates served to provide accurate estimation of randomly distributed motoneurons in NA and XII while minimizing the differences between ages of the standard deviations of nucleoli counted.

5.3.4 Sample Size

Demonstration of patterns of MCD relied on statistical analysis of motoneuron numbers at different developmental stages. Specifically, a Student's t-test was used to compare the mean number of motoneurons at two stages and assess any statistically significant difference. As such, the sample size, i.e., number of animals counted at each stage, largely determined the changes in cell numbers that were able to be resolved. The data from the preliminary studies was used to determine a sample size that would allow accurate assessment of cell death in each nucleus.

In order to determine sample size, the standard deviation for the population and the difference between means to be resolved must be determined. Because the preliminary

Figure 5.5
Standard Deviation After Modification of Sampling Rate
Minimization of Differences Between Ages

$$\sigma = \sqrt{np(1-p)}$$

n = population of sampled nucleoli (=estimated total cells)

p = probability of nucleolus being counted (=1/sampling rate)

	Pilot Study	Proposed Study
Nucleus Ambiguus		
E17	$\sqrt{(3000)(.33)(.66)} = 25.56$	$\sqrt{(3000)(.20)(.80)} = 21.91$
E19	$\sqrt{(2000)(.33)(.66)} = 20.87$	$\sqrt{(2000)(.20)(.80)} = 17.89$
Adult	$\sqrt{(1400)(.20)(.80)} = 14.97$	$\sqrt{(1400)(.33)(.66)} = 17.46$
Hypoglossal Nucleus		
E17	$\sqrt{(4500)(.33)(.66)} = 31.31$	$\sqrt{(4500)(.20)(.80)} = 26.83$
E19	$\sqrt{(3700)(.33)(.66)} = 28.39$	$\sqrt{(3700)(.20)(.80)} = 24.33$
Adult	$\sqrt{(3500)(.20)(.80)} = 23.66$	$\sqrt{(3500)(.33)(.66)} = 27.61$

data relied on two animals per age, standard deviation was estimated from the range of motoneuron counts. Specifically, the range can be considered approximately four times the standard deviation (Mendenhall et.al., 1986). The largest difference between counts was observed in the E17 XII with a low of 4167 cells and a high of 4752 cells. The standard deviation was thus estimated to be 150. The largest standard deviation from the preliminary data was used to provide the most conservative estimate of sample size.

The other factor needed to determine sample size was the difference between means that was deemed useful to resolve. That is, the change in motoneuron numbers between ages that would be considered an important change in population. The preliminary data indicated that MCD may be occurring around E17 and E19. Furthermore, the loss between E17 and E19 was about 35% in NA and just under 20% in XII. With only one age between the two stages (E18), an even distribution of cell loss would imply 17% loss in one day in NA and 10% loss over the same time in XII. Selecting the smaller and more conservative figure, 10% cell loss was deemed the minimal change in cell populations that should be resolved. However, the largest observed number of cells was 4500 in the E17 XII and the smallest observed number 1400 in the adult NA. Thus, to ensure an appropriate sample size, 10% of the smallest number was selected and the minimum difference between cell populations deemed important to resolve was 140 cells.

Determination of sample size can be accomplished by using the formula for the t-statistic and solving for "n" as shown in figure 5.6. For this calculation the standard deviations are the same for both populations. Additionally, use of the same number of animals at each investigated age maximizes the power of the t-test and the formula thus reduces to that shown in 5.6 (2). The equation is then squared (3) and solved for n (4). For many degrees of freedom and nondirectional significance at $P \leq 0.05$, the t-statistic iterates to two. Furthermore by substituting 140 for the difference in means and 150 for the standard deviation a sample size of $n \geq 9.18$ is determined (5). This can be rounded-up

to a sample size of $n=10$. Thus, 10 animals were used to study each nucleus at each developmental stage. In order to eliminate fraternal effects on cell numbers, three different litters were used for the investigation of MCD in prenatal and postnatal day one rats.

Figure 5.6

Derivation of Sample Size

$$\frac{\bar{X}_1 - \bar{X}_2}{\sqrt{\frac{\sigma_1^2}{n_1} + \frac{\sigma_2^2}{n_2}}} \geq t \quad (1)$$

σ = standard deviation n = sample size

$\bar{X}_1 - \bar{X}_2$ = difference between means

$$\frac{\bar{X}_1 - \bar{X}_2}{\sqrt{\frac{2\sigma^2}{n}}} \geq t \quad (2)$$

$$\frac{(\bar{X}_1 - \bar{X}_2)^2 n}{2\sigma^2} \geq t^2 \quad (3)$$

$$n \geq \frac{t^2 2\sigma^2}{(\bar{X}_1 - \bar{X}_2)^2} \quad (4)$$

substitute: $\sigma = 150$; $\bar{X}_1 - \bar{X}_2 = 140$; $t = 2$

$$n \geq \frac{8(150)^2}{(140)^2} = 9.18 \quad (5)$$

Chapter 6

Nucleus Ambiguus: Motoneuron Cell Death

6.1 Introduction

Patterns of respiration, deglutition, and vocalization in the mammalian URT arise, in large part, from the interaction between motoneurons of the nucleus ambiguus (NA) and muscles of the larynx and pharynx. Furthermore, this interaction is modulated by afferent, cortical, and brainstem reticular neurons which synapse on NA motoneurons and, in turn, help to define and refine URT neuromuscular control. Thus, NA motoneurons play a central role in the establishment of precise and coordinated URT neuromuscular activity. Although crucial to the maintenance of normal life, the development of neuromuscular control in the mammalian and particularly in the human URT is poorly understood.

The interaction between cortical and brainstem neurons, motoneurons in NA, and muscles of the larynx and pharynx, must be specific to ensure coordinated and efficient URT control. For example, neurons in the brainstem which modulate patterns of respiration must project, specifically, to those NA motoneurons innervating muscles controlling upper airway patency. This "three-part" specificity (e.g., respiratory neurons to NA motoneurons to URT muscles) is established prenatally, in large part, during and around the period of MCD. Thus, the occurrence of MCD, and its susceptibility to modification by epigenetic signals, may establish a coordinated developmental sequence by which cortical and brainstem neuronal centers can control peripheral URT structures via specific connections with NA motoneurons. That is, those motoneurons establishing specific contacts (i.e., "linking" upper neuronal centers with the proper peripheral muscles) may survive, while those providing "uncoordinated" or improper links would degenerate. Thus, characterization of the course of MCD in NA would define critical periods in the establishment of URT control.

6.2 Experimental Design

6.2.1 Motoneuron Counting in NA

General histological and counting methodologies for the investigation of MCD were defined in Chapter 5. However, the histological appearance and somatotopic organization of the nucleus ambiguus necessitated the addition of a specific counting protocol designed to define clearly the motoneuron population at each developmental stage. That is, a protocol was designed to count the same group of motoneurons at each age thus ensuring that any observed changes in cell numbers were due to MCD rather than the inclusion or exclusion of counted cells.

The main concern in counting NA was over-estimation of cell numbers by inclusion of non-URT motoneurons. This was an issue due to the ventrolateral brainstem location of NA placing it in close proximity to the facial and reticular nuclei. Additionally, NA itself contains many parasympathetic efferent neurons which could possibly interfere with the assessment of URT motoneurons. Thus, several experimental parameters were adapted and used for counting cells at all investigated stages. 1) Motoneuron counts in NA began at E17 when the nucleus first appeared *fully* distinct (see Results). This allowed greater assessment of the boundaries of NA and identification of surrounding nuclei thus minimizing confusion between cell populations. 2) Only motoneurons in the compact formation of NA were counted. This attempted to restrict counts to URT motoneurons while minimizing the counting of parasympathetic neurons from the external formation of NA (see Chapter 2). 3) Rostral counts were started at the caudal-most border of the facial nucleus. This established a constant point, found at all ages studied, along the rostral-caudal axis from which cell counts could be initiated. Additionally, in less mature organisms, this prevented the counting of motoneurons in the superior pole of the facial nucleus which often extended dorsally in-line with NA. 4) Caudally, NA was often diffuse and represented by few cells thus making assessment of

the precise caudal pole of NA highly subjective. As such, counts were terminated at the pyramidal decussation, often defined as the end of NA (Bystrycka and Nail, 1985; Yoshida et.al., 1984). This established a universal and objective "stop-point" for the taking of cell counts at all ages. 5) The precise position of the NA column of motoneurons with regard to the center and edges of the brainstem was continuously assessed with an ocular micrometer. This allowed the position of the nucleus to be determined in all serial sections, particularly toward the caudal pole where the loose formation was less clearly defined.

Aside from the above methodologies, the counting of motoneurons in NA required vigilant attention to the rostral-caudal changes in brainstem morphology surrounding NA. In particular, two nuclei, the linear and lateral reticular nuclei, appeared in close proximity to NA near its rostral and caudal poles, respectively. These nuclei, more so in the prenatal brainstems, contained large cells with distinct nucleoli which often obfuscated the smaller, less distinct NA. This was less of a problem with the lateral reticular nucleus which could be defined as it emerged ventrally and then ventrolaterally along the caudal part of NA. The linear reticular nucleus, however, appeared rostrally, ventrolateral to NA, and caudally, dorsolateral to NA. Midway between its two extremes, the linear nucleus was directly lateral to NA, at a point when the rostral-caudal column of cells representing NA becomes thin and less distinct. Thus, on initial inspection of a section through the middle of NA, the linear nucleus could have been mistaken for NA. However, this was avoided by tracing NA in serial sections from rostral to caudal with an ocular micrometer, thus charting its position from the most distinct toward the more diffuse morphologies. Furthermore, immediately lateral to NA perforating capillaries separating NA from the linear nucleus have been consistently observed. This provided an additional landmark for distinguishing NA from the linear nucleus.

These specific protocols for the investigation of MCD in NA were necessary because of the caudally diffuse appearance of the nucleus, the presence of parasympathetic neurons in the nucleus, and the proximity of NA to surrounding reticular and motor nuclei in the ventrolateral brainstem. These protocols were designed to minimize the inclusion of extraneous cell populations in the counts of NA motoneurons while allowing a complete assessment of those motoneurons projecting to the URT.

6.2.2 Materials and Methods

Developmental changes in brainstem morphology were assessed as early as E13, however, counts of NA motoneurons were made in E17, E18, E19, E20, P1 and adult rats. Counts began at E17 as it was the first age at which NA was clearly defined and thus the earliest stage at which reliable motoneuron counts could be obtained. Counts were made at each successive prenatal age until results indicated the end of MCD by comparison with postnatal motoneuron counts. As indicated in Chapter 5, 10 animals per age were counted representing three different litters. Although each litter was typically represented by no less than three rats, insufficiently processed brainstems resulted in two litters, one at E17 and one at P1, being represented by two rats. All specimens were coded by age, litter, and order removed from the uterine sac. As such, "E17-A3", would be the third rat of litter "A" removed from a female at the 17th day of gestation. The final sample population is not in sequential order, and gaps in specimen numbers reflect tissue damaged at later stages of processing or paraffin blocs not yet sectioned.

Prenatal rats were removed from commercially bred females and thus weight and crown-rump lengths were taken to ensure accuracy in assessment of gestational age. Results of these measurements are shown in table 6.1 and graphically in figure 6.1. They demonstrate the logarithmic growth of the prenatal rat over the period studied. Further, the highly statistically significant differences between the measurements at each stage enabled animals to be aged with a great degree of accuracy. The day of a positive vaginal smear was considered E0 and birth occurred on day E22, which was also

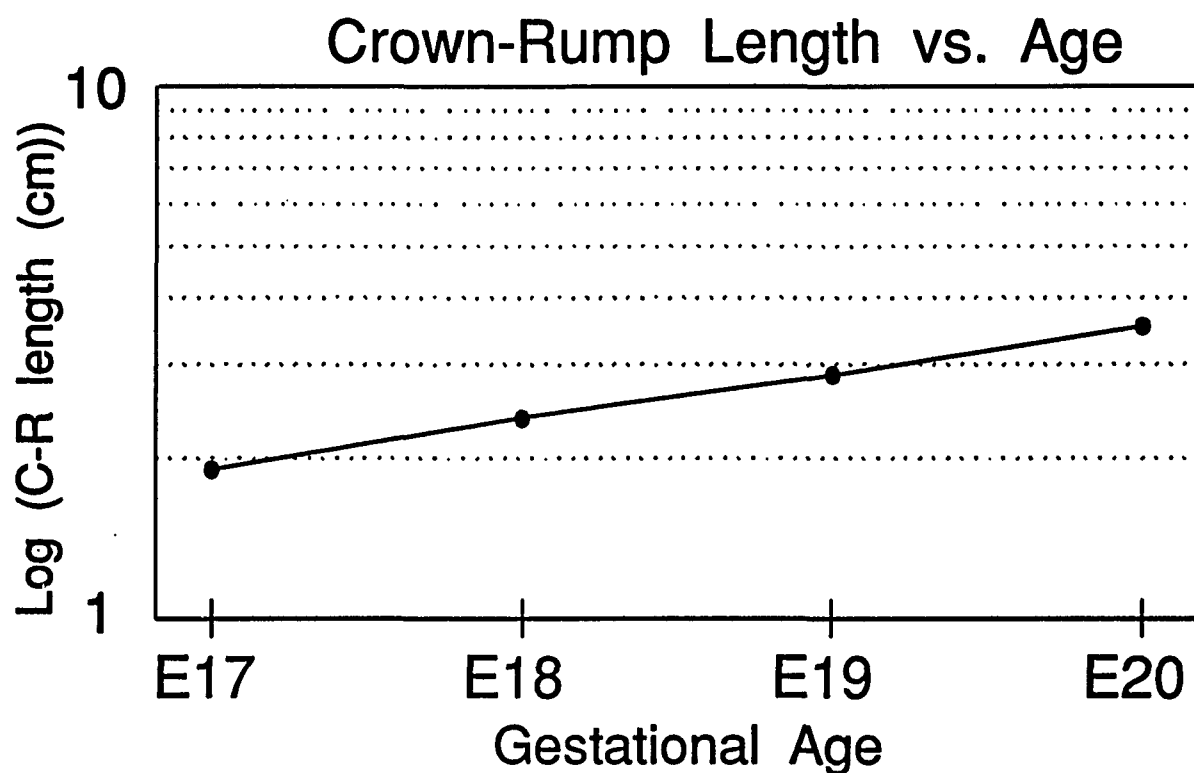
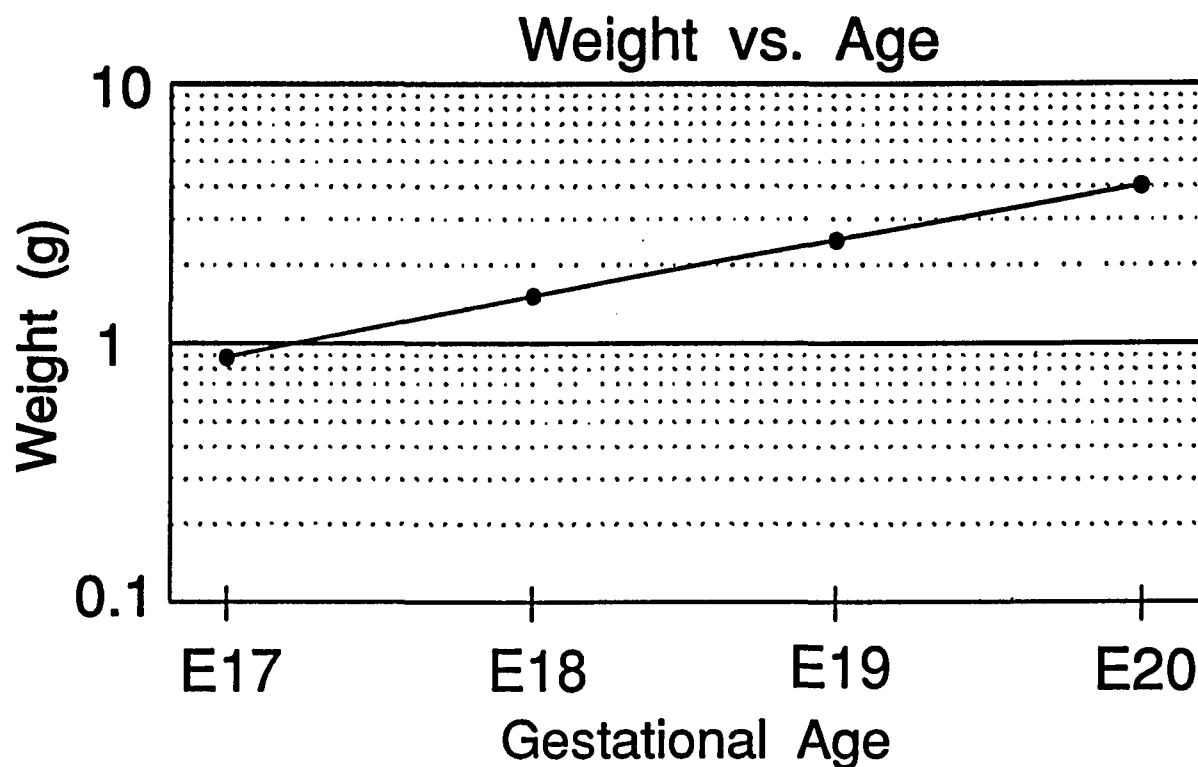
Table 6.1: Weights and Crown-Rump Lengths of Rats Used For the Assessment of MCD in NA

E17	Weight (g)	C-R Length (cm)	E20	Weight (g)	C-R Length (cm)
E17-A3	1.0095	---	E20-A1	4.0567	3.725
E17-A4	.9404	---	E20-A2	4.3367	3.783
E17-A5	.9118	---	E20-A6	3.8955	3.579
E17-A6	.9766	---	E20-B2	4.4331	3.642
E17-B5	.9159	---	E20-B3	3.9326	3.484
E17-B6	.8900	---	E20-B4	3.6537	3.383
E17-C2	.8193	1.912	E20-B5	4.0231	3.422
E17-C3	.7778	1.879	E20-C2	4.0168	3.485
E17-C4	.8292	1.97	E20-C4	4.0195	3.408
E17-C5	.8195	1.872	E20-C5	4.1421	3.469
Mean	.8890	1.909	Mean	4.0510	3.538
SEM	.0253	.022	SEM	.0733	.046
E18	Weight (g)	C-R Length (cm)	P1	Weight (g)	C-R Length (cm)
E18-D2	1.3005	---	P1-E2	6.1878	---
E18-D4	1.3091	---	P1-E5	6.1724	---
E18-D5	1.1715	---	P1-F1	8.1955	---
E18-E2	1.6206	2.381	P1-F2	8.6615	---
E18-E4	1.6304	2.346	P1-F3	7.015	---
E18-E5	1.6167	2.353	P1-F4	7.7683	---
E18-E6	1.7548	2.305	P1-G3	6.7144	---
E18-F3	1.5584	2.505	P1-G5	5.7836	---
E18-F4	1.7632	2.492	P1-G6	6.5673	---
E18-F5	1.4459	2.341	P1-G7	6.7715	---
Mean	1.5171	2.389	Mean	6.9837	N/A
SEM	.0673	.030	SEM	.0313	N/A
E19	Weight (g)	C-R Length (cm)	Adult	Weight (g)	C-R Length (cm)
E19-D1	2.1702	2.745	A-8	600	---
E19-D2	2.3023	2.757	A-9	685	---
E19-D4	2.4978	2.777	A-10	635	---
E19-E3	2.4568	2.759	A-11	645	---
E19-E5	2.6380	2.964	A-12	735	---
E19-E6	2.5572	2.867	A-15	426	---
E19-F1	2.4202	2.988	A-16	358	---
E19-F2	2.4117	2.892	A-17	354	---
E19-F4	2.7170	2.916	A-18	354	---
E19-F7	2.8689	2.848	A-19	330	---
Mean	2.5040	2.861	Mean	512.2	N/A
SEM	.0675	.028	SEM	53.79	N/A

(N/A = not applicable)

Figure 6.1

Weight and Crown-Rump Length Versus Age in Rats Used to Assess MCD in NA



considered P1. All P1 rats studied were sacrificed and processed for cell counts within three hours of birth. Adult rats were approximately six months of age.

6.3 Results

The investigation of the brainstem in a pre- and postnatal series of rats has provided insight into developmental phenomena in the medulla, including: 1) gross and histological changes in overall brainstem morphology prior to, and including, the period of MCD; 2) differentiation of the brainstem into discrete neuronal pools, in particular, the formation of the nucleus ambiguus (NA); and 3) maturational changes within NA, including the occurrence of MCD. The results of this investigation are shown in figures 6.2-6.16 and table 6.2.

6.3.1 Morphology

The earliest ages studied, i.e., E13-E15, represent the final stages of neurogenesis as indicated by dramatic increases in brainstem size, decreases in the number of mitotic profiles, and reduction of the size of the proliferative zone (figures 6.3-6.7). At E13 the brainstem was extremely narrow in the dorsoventral plane and is shown *in situ* as it was too small to be removed and then adequately embedded in paraffin for sectioning (figure 6.2). The glossopharyngeal and vagus nerves were already evident projecting from the brainstem near their mature ventrolateral position. Associated with the nerves was a prominent sensory ganglion, the jugular ganglion of either the ninth or tenth nerve. Along the entire dorsal aspect of the brainstem was a thick, densely staining region of recently generated, differentiating and migrating neuroblasts called the proliferative zone. On higher power this region was seen to be composed of many large nucleated cells, particularly along the medial border of the ventricle at the basal plate (figure 6.3). These represented newly formed motoneurons, some of which were beginning to migrate to their mature location in the brainstem (see chapter 9 for further studies on this

developmental event). At the ventricular layer of the proliferative zone additional motoneurons continued to be formed as evidenced by the numerous mitoses seen in the ependyma.

By E14 the brainstem grew sufficiently so as to allow it to be embedded and sectioned in isolation. However, aside from size (N.B. photographs at different magnifications), the brainstem underwent little change in overall morphology (figure 6.4). The characteristic "flattened" appearance persisted as did the presence of a thick, densely staining proliferative zone. On higher power this zone seemed to have reduced in size and the large pools of newly formed motoneurons were no longer as apparent (figure 6.5). The number of mitoses along the ventricle diminished with the majority of profiles being found more laterally at the alar plate.

A dramatic change in brainstem morphology was observed between E14 and E15. The E15 brainstem increased in breadth along the dorsoventral plane, particularly in the lateral region (figure 6.6). The ventricular layer was no longer as apparent and significantly reduced in size. The remainder of the brainstem began to appear less homogeneous indicating differentiation into discrete neuronal populations. Mitotic activity along the basal plate had stopped with infrequent profiles seen primarily in the extreme lateral portions of the ventricular layer (figure 6.7).

Subsequent changes in the morphology of the brainstem largely related to size and further differentiation of discrete motor populations. By E17 mitotic activity along the entire ventricle stopped and the proliferative zone was no longer present. The brainstem had achieved a heterogeneous appearance characteristic of the mature nervous system with well differentiated subpopulations of neurons. Little gross anatomical changes were apparent through birth while the adult brainstem exhibited a distinct morphology.

6.3.2 *Nucleus Ambiguus Formation*

The mature NA is found in the ventrolateral region of the brainstem and it is in this location that sections were examined for the assessment of nucleus formation. Figures 6.3 and 6.5 show the ventrolateral portion of E13 and E14 brainstems, respectively. These sections were taken at the level of the rostral compact formation of NA and no apparent nucleus was present. It was not until E15 that large nucleated neuroblasts were first observed clustering in the mature location of NA (figures 6.7, 6.8 and 6.10). This was only observed in some specimens, and within individual brainstems, the nucleus was inconsistently found in all rostral to caudal sections. When found, however, the nucleus appeared diffuse and ill-defined. By E16 clusters of neuroblasts in the region of NA were consistently found in all specimens (figures 6.9 and 6.11). However, as in E15 brainstems, the nucleus was not present in all sections through the caudal extent of NA. The E16 NA appeared more defined, but did not exhibit a consistently distinct appearance allowing the establishment of a compact formation in all specimens or levels of the brainstem.

By E17, NA appeared fully distinct, was present in all specimens, and could be found in all rostral to caudal sections throughout its extent (figure 6.12). The motoneurons in NA at this stage retained their immature neuroblastic appearance and, in the rostral compact formation, were densely clustered sometimes overlapping in the thin 8µm sections. No significant changes were observed at E18, however, by E19 motoneurons in NA began to exhibit more mature morphological features (figure 6.13). For example, cells began to exhibit a densely staining cytoplasm with the characteristic "triangular" shape of postnatal motoneuronal cell bodies. At this stage, the nucleus appeared less dense than at E17, but was more distinct and was readily apparent in the majority of sections through the medulla.

Examination of postnatal tissue indicates little gross morphological change in NA after E19 (figures 6.14 and 6.15). That is, the late gestation and postnatal NA differed

little in apparent density of cells or in somatotopic organization of the compact formation. However, on a neuronal level, by P1 all cells in NA exhibited classic motoneuron morphology and showed an increased affinity for the Nissl stain cresylviolet. This affinity for Nissl stain increased through adulthood in which NA neurons stained particularly strongly for cresylviolet giving the nucleus a prominent appearance (compare figures 6.14 and 6.15).

6.3.3 Motoneuron Cell Death

Results of counts made of motoneurons in NA between E17 and adulthood are shown in table 6.2 and graphically in figure 6.16. The nucleolar counts per section per specimen are compiled in Appendix B. At E17 there were 2747 ± 92 (average $\pm$ SEM) motoneurons in NA. The number of cells decreased to 2589 ± 101 at E18 but the difference in motoneuron populations between these ages was statistically significant only for a uni-directional t-test at $P \leq .05$. A further decrease in motoneuron numbers was seen through E19 where 2061 ± 60 cells were counted. Calculation of the t-statistic for a non-directional test, in comparing counts at E18 and E19, indicated a highly significant loss of cells ($P \leq .001$). Significant motoneuron losses were also seen through E20 where 1630 ± 79 cells were counted ($P \leq .001$), and subsequently through P1 (1343 ± 54 motoneurons; $P \leq .001$). No further loss of cells was observed between P1 and adult.

While quantitative analysis of motoneuron populations could only be accomplished after the nucleus was fully distinct, the occurrence of MCD could be assessed qualitatively at earlier stages by examining NA for degenerating cells. There were no degenerating neurons seen in NA at E15, however, some apoptotic cells were seen at E16. These were few in number and observed only in occasional sections through NA. In contrast, degenerating cells were frequently found in NA at E17, E19 and E20. At E18, some apoptotic cells were found in NA, but not as often as at other ages. In the P1

Figure 6.2: Transverse section through an E13 brainstem at the level of NA. NA was not seen at this stage. The emerging vagus and glossopharyngeal nerves (arrow on left side), however, indicate that this is the level at which ambigual motoneurons are located. Shown in this figure is the fourth ventricle (v) for purposes of orientation. For further orientation see figure 2.2 and chapter 4. (H&E, 80x)

Figure 6.3: Closer view of the left side of an E13 brainstem at the level of NA. The thick, dense staining region indicated by the star is the proliferative zone. Numerous mitoses (e.g., arrow) are seen along the fourth ventricle indicating the generation of cells during this period. At this stage, no cluster of neurons representing NA were observed. (H&E, 200x)

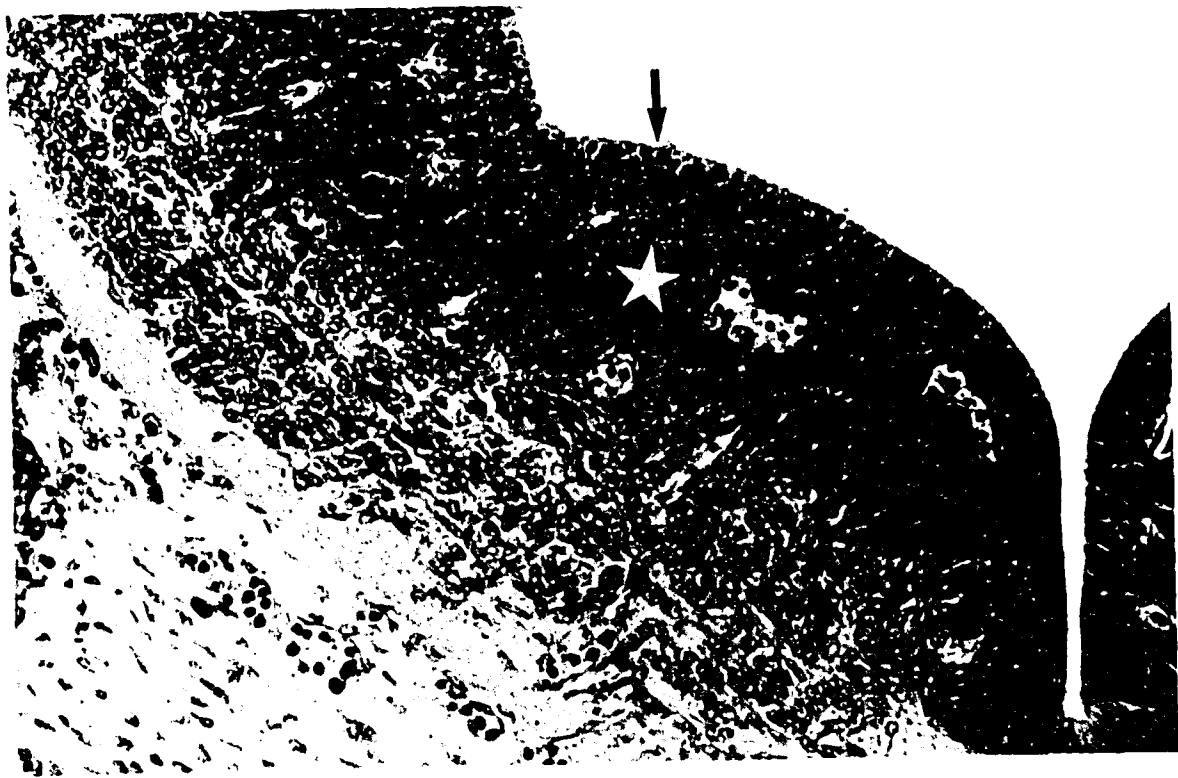


Figure 6.4: Transverse section through an E14 brainstem at the level of NA. This section is oriented as the E13 brainstem in figure 6.2 but photographed at a lower magnification. The level of NA is indicated by the presence of the vagus and glossopharyngeal nerves emerging from the lateral brainstem (arrow indicates left nerves). (H&E, 40x)

Figure 6.5: Closer view of the left side of above E14 brainstem. The proliferative zone (star) has greatly reduced in size and fewer mitoses are apparent along the ventricle (e.g., arrow). As in the E13 brainstem no cluster of cells defining NA could be observed in the ventrolateral region. (H&E, 125x)

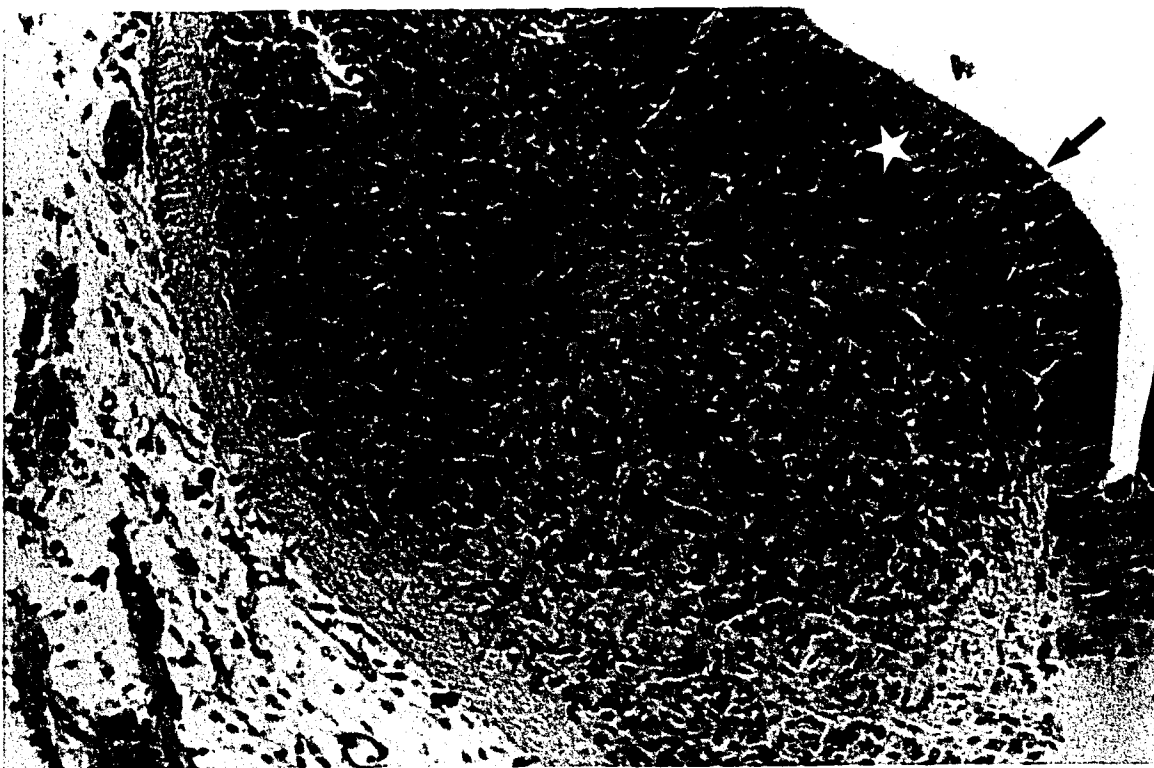
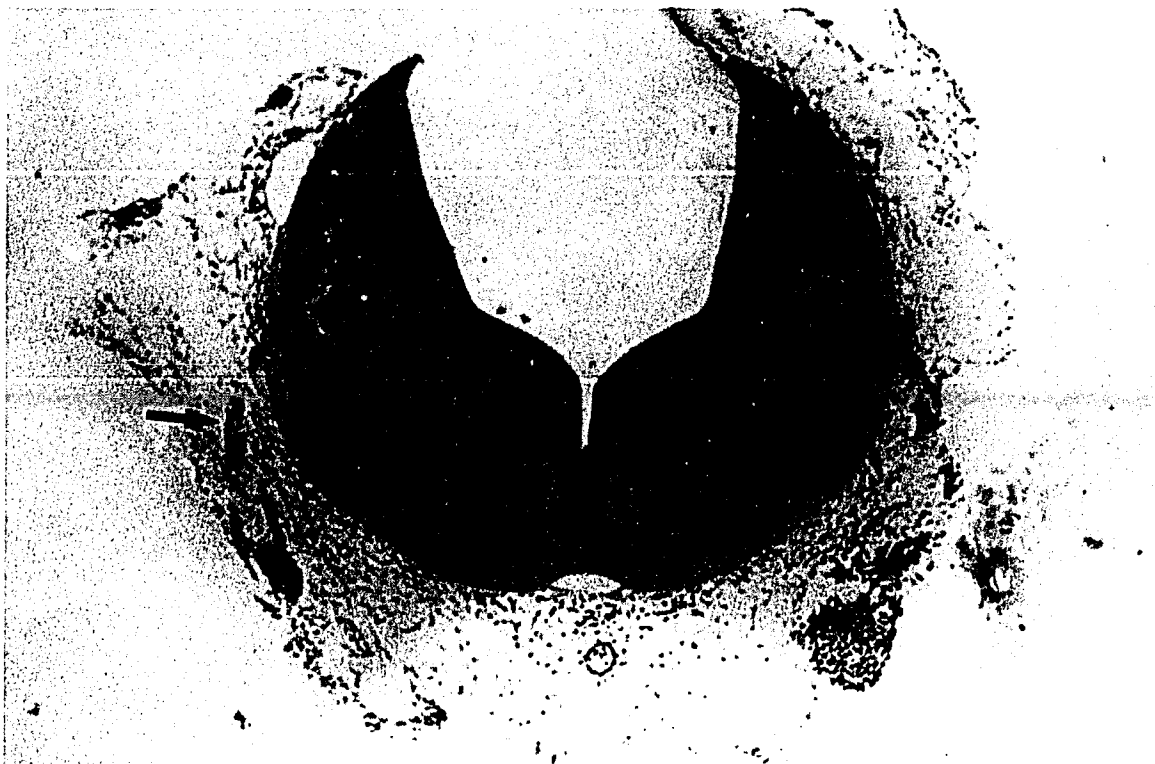


Figure 6.6: Transverse section through an E15 brainstem at the level of NA. As in the previous series the level was identified by the vagus and glossopharyngeal nerves emerging from the lateral brainstem (left nerve indicated by arrow). Although no considerable change in brainstem size was noted between E14 and E15, the morphology of the brainstem changed dramatically (compare to figure 6.4). (H&E, 40x)

Figure 6.7: Closer view of the left side of the above E15 brainstem. At this stage the proliferative zone is almost gone (star) and no mitoses are seen along the ventricle. In the ventrolateral brainstem, for the first time, NA appears as a diffuse cluster of cells (arrow). (H&E, 125x)

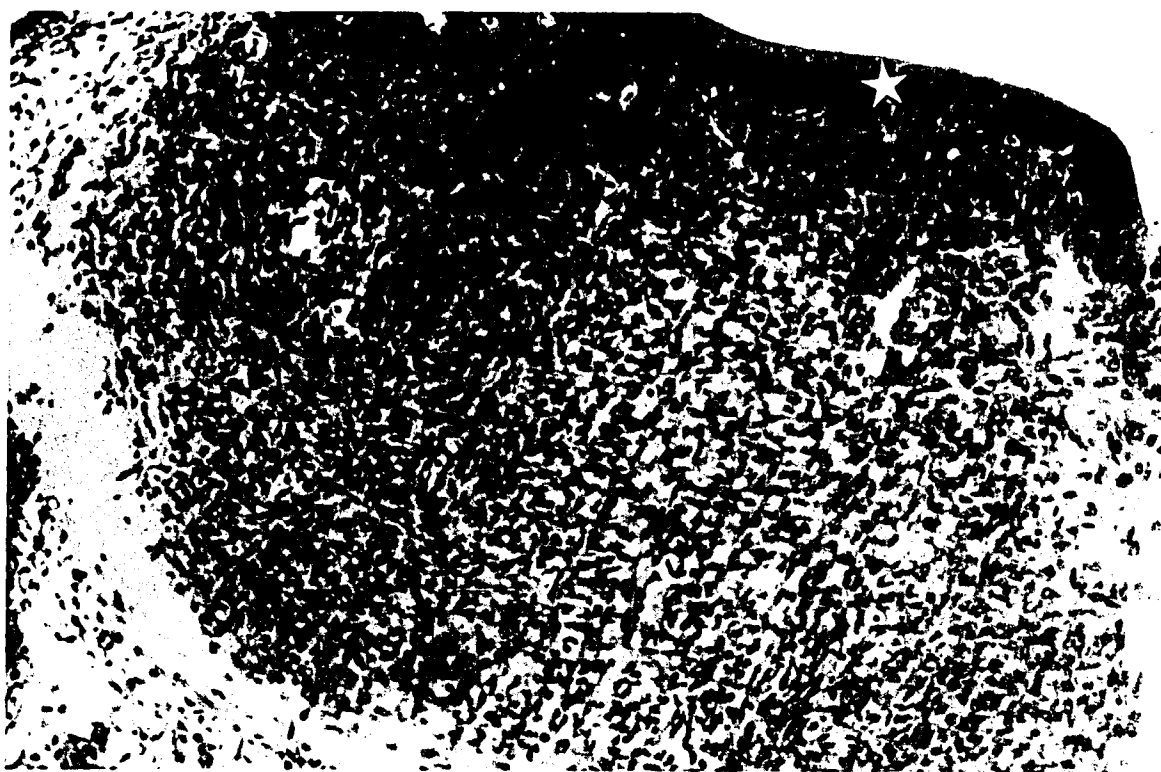
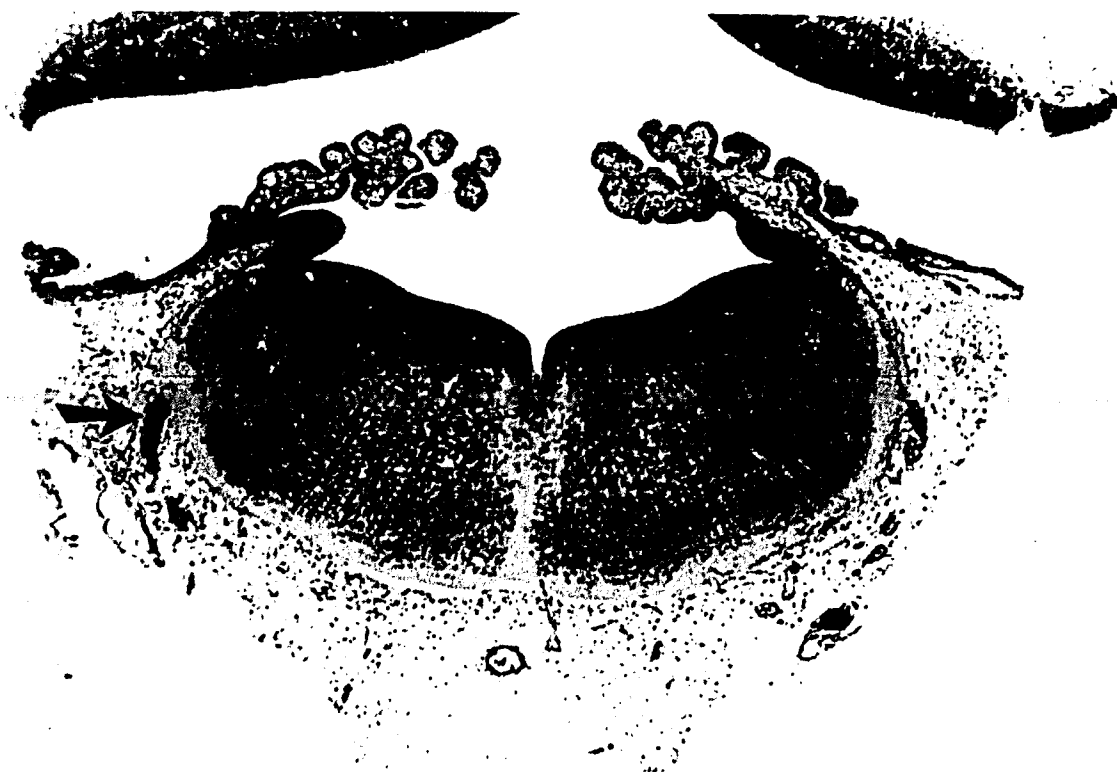


Figure 6.8: NA on the left side in an E15 brainstem (between arrows). Although diffuse in appearance, the nucleus is clearly forming in the mature location. Although NA was found in this section, it was not apparent in all sections through the nucleus and was not found in all E15 specimens. (H&E, 200x)

Figure 6.9: NA on the left side in an E16 brainstem (between arrows). At this stage the nucleus appears more distinct and was found in all specimens, although not in all serial sections. (H&E, 200x)

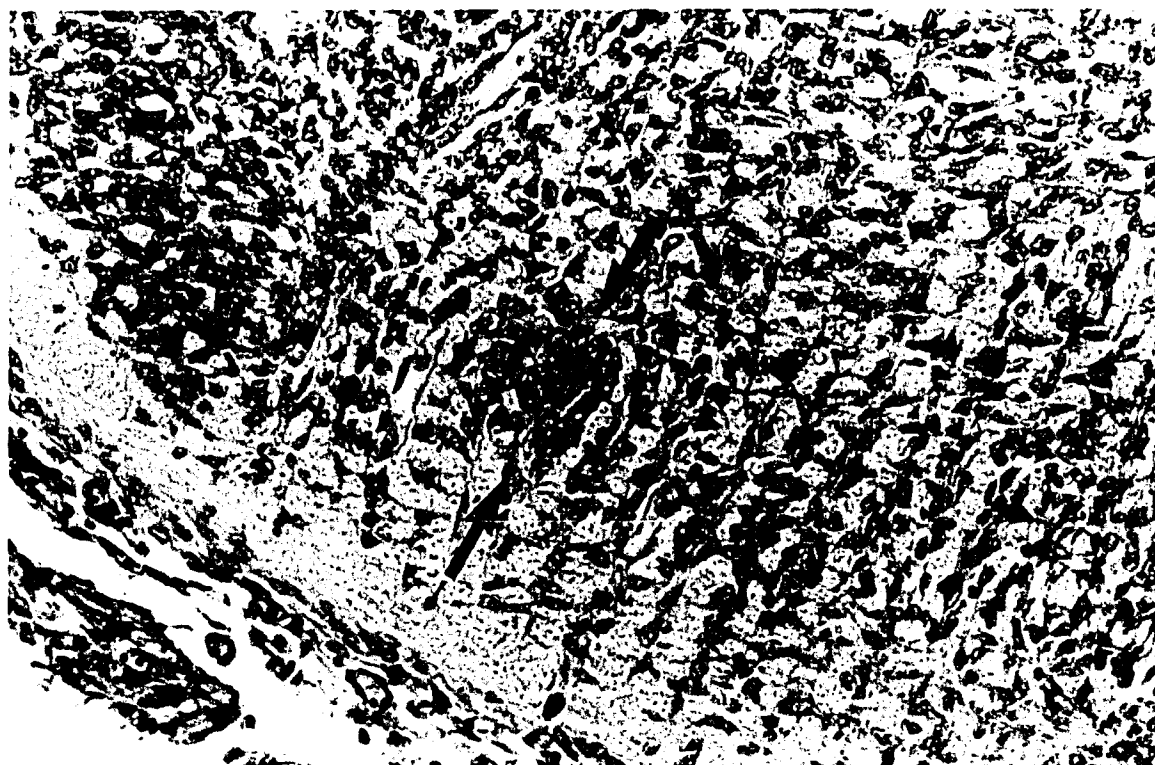


Figure 6.10: This is a closer view of the E15 NA (between arrows) depicted in figure 6.8. It appears as a cluster of similar cells exhibiting clear nuclei and distinct nucleoli. The density of the nucleus often causes cells to overlap in the microscopic field. (H&E, 310x)

Figure 6.11: This is a closer view of the E16 NA (arrow) depicted in figure 6.9. The nucleus is well-defined and cells exhibiting distinct nucleoli are apparent (e.g., near arrow tip). Motoneuron counts were not taken at this stage as the nucleus was not apparent in all serial sections. (H&E, 310x)



Figure 6.12: NA in an E17 rat brainstem. The nucleus is clearly defined and distinct nucleoli are apparent in the motoneurons. Cell counts were initiated at this stage and indicated approximately 2700 motoneurons in the nucleus (see text for further details). (H&E, 310x)

Figure 6.13: NA in an E19 rat brainstem. The nucleus appears darker at this stage as the motoneurons begin to exhibit adult like morphology with greater cytoplasmic staining. Cell counts at this stage indicated approximately 2000 motoneurons in NA, less than at E17. See text for further details on the course of motoneuron cell death in NA. (H&E, 310x)

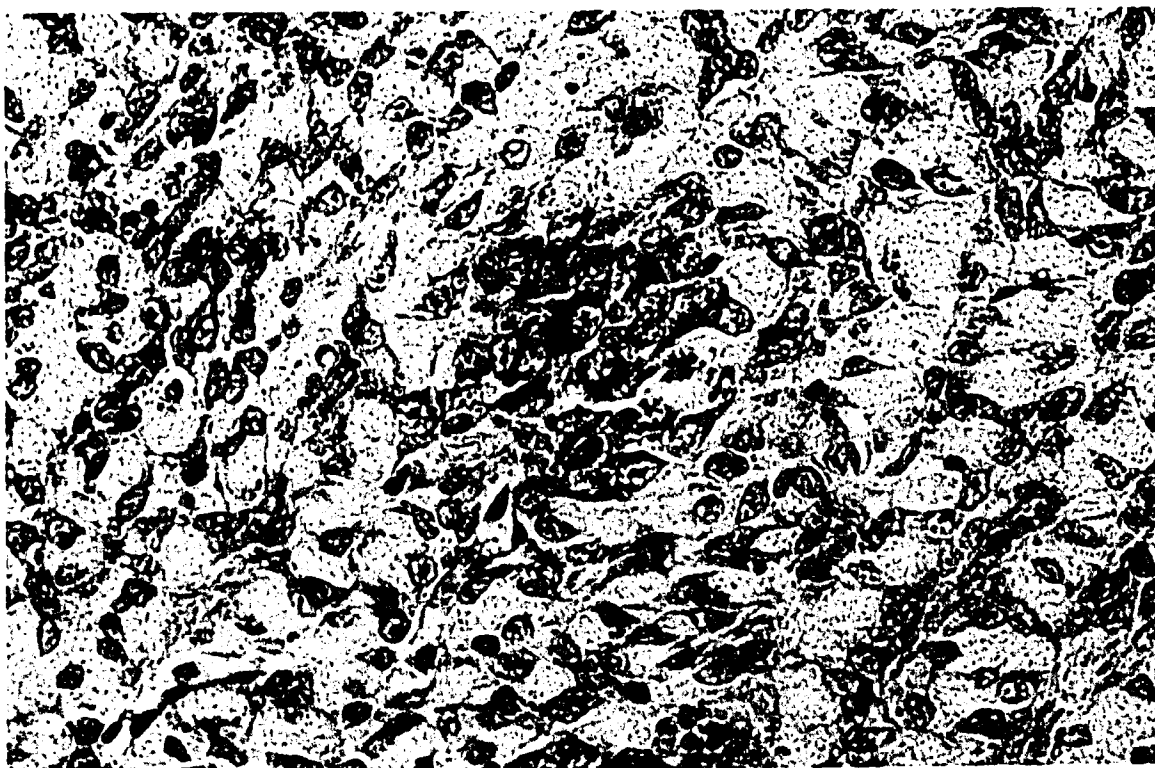
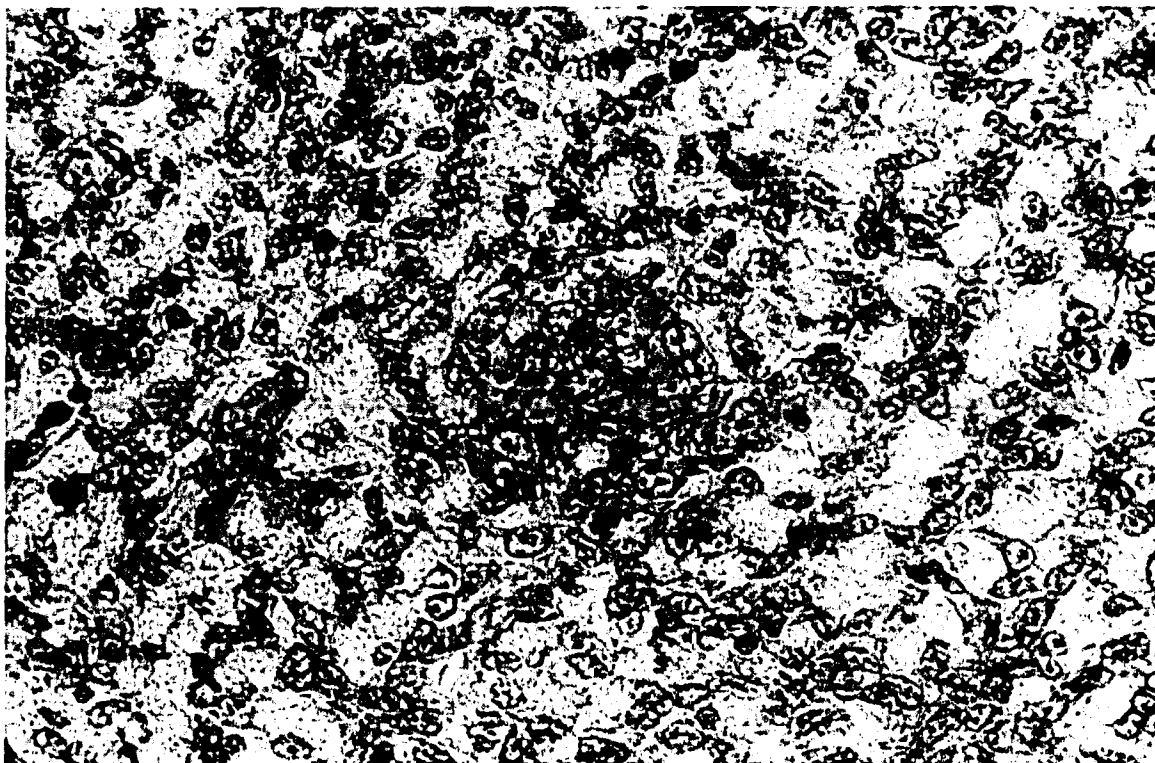


Figure 6.14: NA in a P1 rat brainstem. The nucleus, although appearing less distinct than at E19 (see figure 6.13), is clearly defined (arrows). Cells at this stage show adult-like morphology (i.e., somewhat "triangular" in shape, e.g., near lower right arrow-tip) and exhibit distinct nucleoli. Motoneurons counts showed about 1350 cells in the newborn NA. (cresylviolet, 200x)

Figure 6.15: NA in an adult rat brainstem. The nucleus is clearly defined by densely staining motoneurons and prominent nucleoli are apparent. Counts at this stage were not statistically different from those at P1 indicating that no cell death occurred postnatally in NA (see text for discussion). (cresylviolet, 200x)

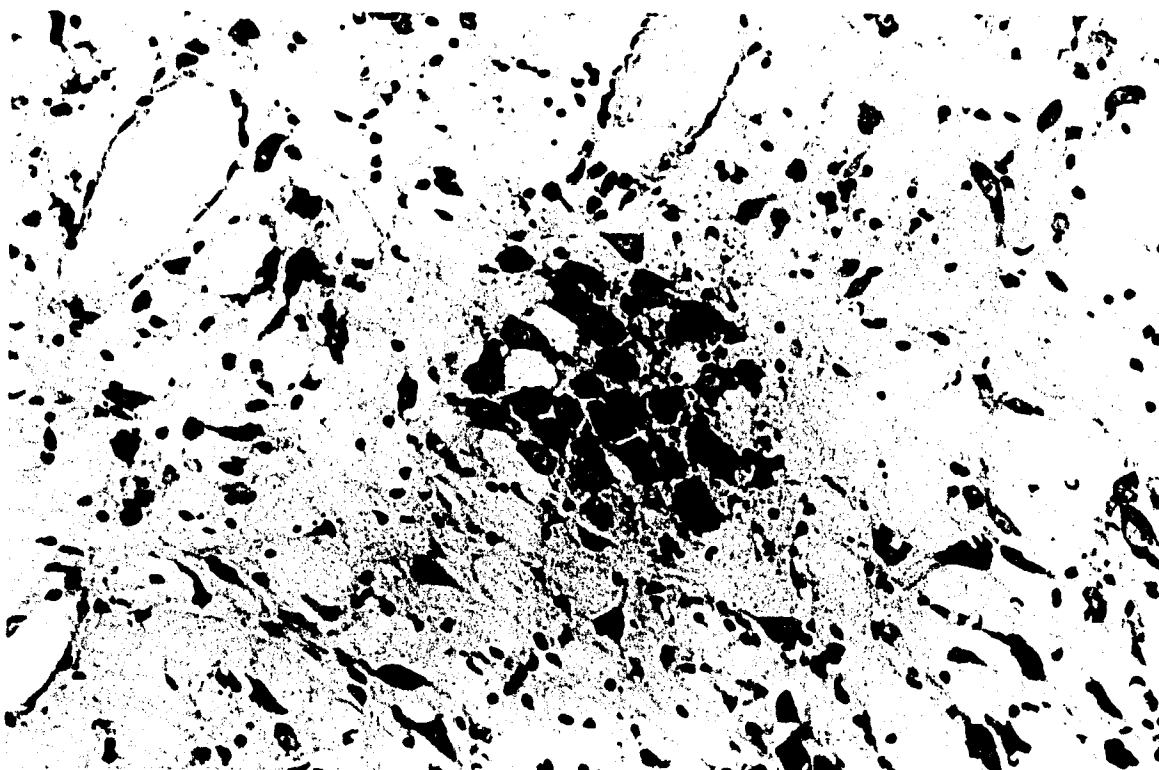
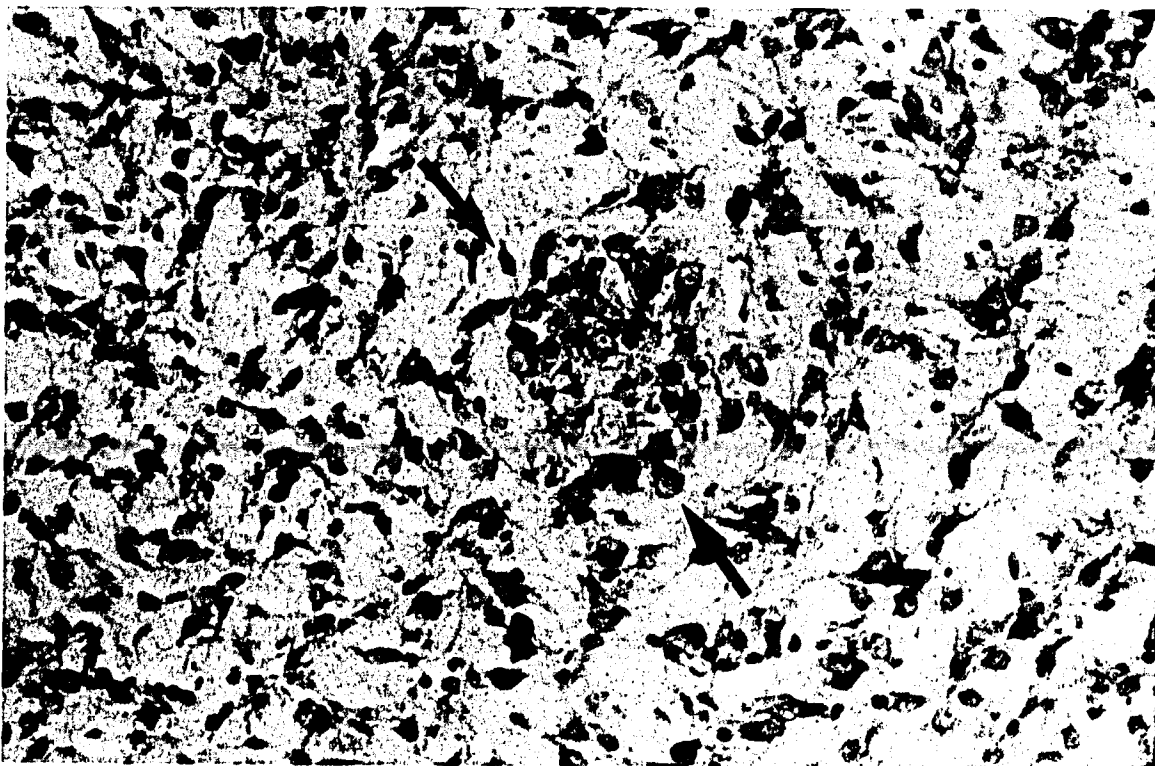


Table 6.2: Estimated Total Cells in the Nucleus Ambiguus

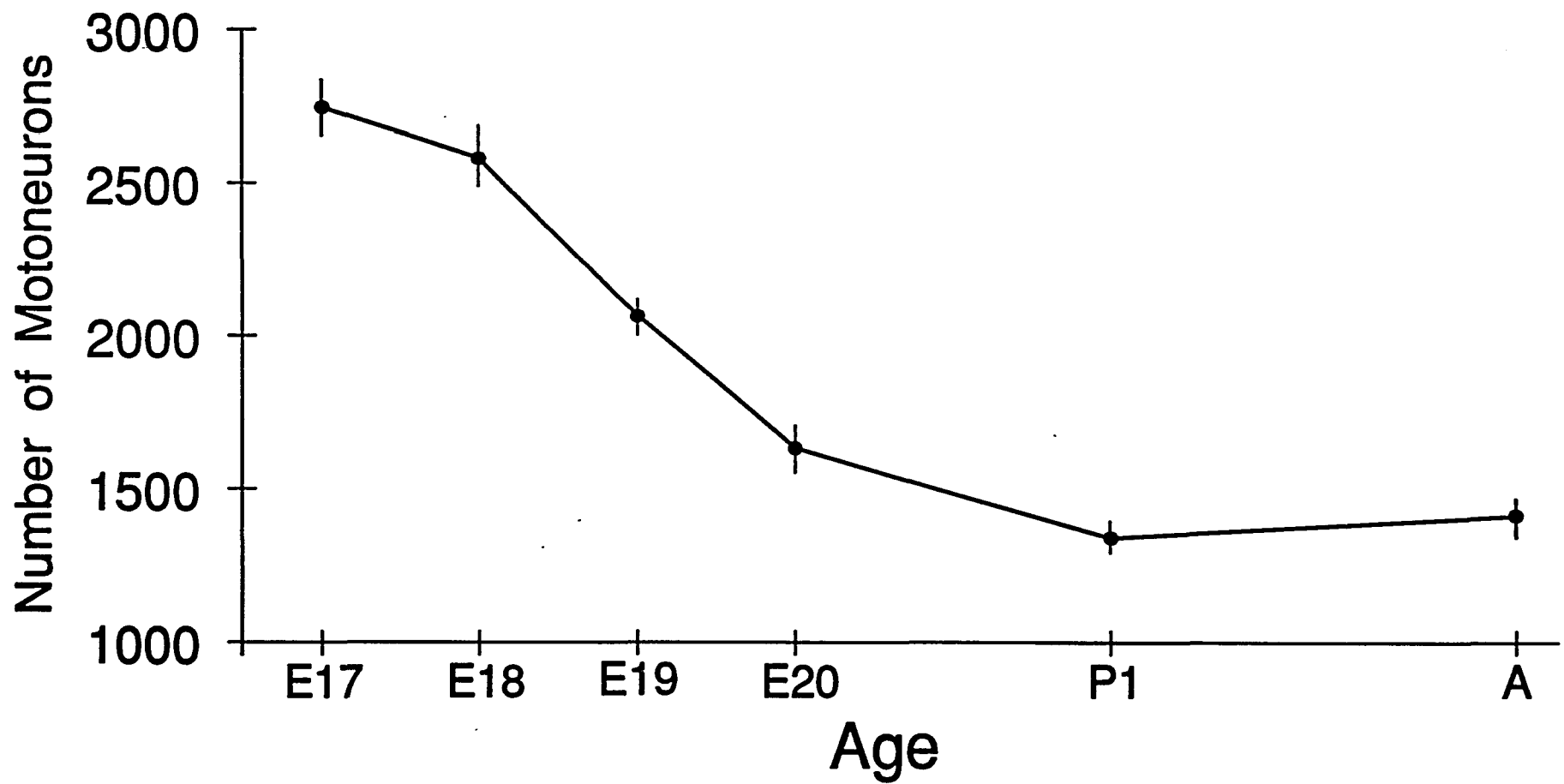
E17	Left	Right	Average	E20	Left	Right	Average
E17-A3	2625	2670	2647.5	E20-A1	1605	1685	1645
E17-A4	2805	2975	2890	E20-A2	1730	1620	1675
E17-A5	3310	3095	3202.5	E20-A6	1735	2020	1877.5
E17-A6	2825	3230	3027.5	E20-B2	1840	1890	1865
E17-B5	2920	2635	2777.5	E20-B3	1545	1675	1610
E17-B6	2260	2400	2330	E20-B4	1820	2055	1937.5
E17-C2	2795	2715	2755	E20-B5	1600	1470	1535
E17-C3	2505	2455	2480	E20-C2	1160	1190	1175
E17-C4	2590	2280	2435	E20-C4	1350	1375	1362.5
E17-C5	2995	2855	2925	E20-C5	1550	1675	1612.5
Mean	2763	2731	2747	Mean	1593.5	1665.5	1629.5
SEM	96.41	102.96	92.41	SEM	70.48	91.57	78.69

E18	Left	Right	Average	P1	Left	Right	Average
E18-D2	1965	2415	2190	P1-E2	1265	1180	1222.5
E18-D4	2470	2605	2537.5	P1-E5	1435	1305	1370
E18-D5	2520	2460	2490	P1-F1	1290	1370	1330
E18-E2	2605	2575	2590	P1-F2	1640	1550	1595
E18-E4	2510	2565	2537.5	P1-F3	1340	1515	1427.5
E18-E5	2970	3225	3097.5	P1-F4	1455	1555	1505
E18-E6	2690	3180	2935	P1-G3	1385	1415	1400
E18-F3	2050	2135	2092.5	P1-G5	1240	1295	1267.5
E18-F4	2580	2815	2697.5	P1-G6	1090	925	1007.5
E18-F5	2625	2810	2717.5	P1-G7	1350	1265	1307.5
Mean	2498.5	2678.5	2588.5	Mean	1349	1337.5	1343.25
SEM	98.14	112.64	101.07	SEM	48.96	64.22	53.87

E19	Left	Right	Average	Adult	Left	Right	Average
E19-D1	2170	1930	2050	A-8	1557	1599	1578
E19-D2	1760	1785	1772.5	A-9	1878	1830	1854
E19-D4	2255	2325	2290	A-10	1362	1299	1330.5
E19-E3	2210	2130	2170	A-11	1248	1245	1245.5
E19-E5	2195	1855	2125	A-12	1533	1536	1534.5
E19-E6	2235	2120	2177.5	A-15	1314	1287	1300.5
E19-F1	1725	1740	1732.5	A-16	1338	1296	1317
E19-F2	2155	2085	2120	A-17	1227	1149	1188
E19-F4	2035	2385	2210	A-18	1323	1449	1386
E19-F7	2010	2120	2065	A-19	1359	1284	1321.5
Mean	2075	2047.5	2061.25	Mean	1413.9	1397.4	1405.65
SEM	64.18	72.35	60.47	SEM	65.00	68.46	65.93

Figure 6.16

Estimated Number of Motoneurons Versus Age in NA:
Graphical Depiction of the Course of MCD



NA, dying cells were observed very infrequently, however, these may have been small glial cells as Nissl stains do not clearly distinguish degenerating cells.

6.4 Discussion

The nucleus ambiguus (NA) contains motoneurons which innervate muscles of the larynx and pharynx. As such, this brainstem motor nucleus plays a critical role in the establishment of upper respiratory tract (URT) patterns of respiration, deglutition and vocalization. While these URT activities require specific neuromuscular interactions between brainstem and periphery, little is known of the prenatal development of NA, nor of critical periods in the formation of URT neuromuscular specificity and control. This study has investigated the prenatal development of NA and has defined gestational periods during which ambigual motoneurons may be generated and migrate; are organized into a coherent nucleus; and undergo specific maturational events, including naturally occurring cell death.

Observations of ependymal layer mitotic activity and changes in overall brainstem morphology indicated the occurrence of neurogenesis between E13 and E15 (figure 6.2 to 6.7). During this period numerous mitotic profiles were observed in the ventricular layer, implying proliferation of motoneurons at the basal plate. This observation is supported by the thymidine labeling studies of Altman and Bayer (1980) who indicated E14 as the peak generation time of rat NA motoneurons. At E13 and E14 these neuroblasts appeared densely clustered along the dorsal brainstem and migration of these cells out of the proliferative zone, and into discrete, less dense neuronal centers, would account for the dramatic change in brainstem morphology seen at E15. Such migration and reorganization of neuroblasts is further supported by the heterogeneous appearance of the brainstem, first observed at E15. A heterogeneous, rather than homogeneous, staining brainstem implies the separation of neurons into discrete functional pools by this time. Thus, these results indicate a temporal sequence of neurogenesis, migration and

organization with the first two occurring around E13 and E14, and the latter around E15. That some, albeit fewer, mitotic profiles were seen at E15 and E16 indicates some overlap in the periods of neurogenesis and organization into discrete neuronal pools.

While heterogeneity of brainstem staining implied organization of the brainstem into discrete neuronal pools by E15, closer inspection provided direct evidence for such a pattern of development. Examination of the ventrolateral brainstem showed no apparent NA at E13 or E14 (figures 6.3 and 6.5), but did find an ill-defined NA at E15 (figure 6.7 and 6.8). NA became more distinct in appearance through E16, becoming fully distinct throughout its full extent at E17. Whether this increase in distinction was due to the migration of additional motoneurons to NA, differentiation of neuroblasts within NA, or biochemical changes affecting staining of individual cells could not be determined by routine histology. Chapter 9 will present studies using neural tracers which indicated that the changes in NA could not be attributable to motoneuron migration during this period. The appearance of NA at later stages of gestation was similarly distinct and motoneurons began to exhibit a more mature morphology by E19. As such, these results indicated the generation of NA motoneurons through E14, organization of a distinct nucleus between E15 and E17, and maturation and further differentiation of the neuroblasts through E19. This overall temporospatial pattern observed in the rat NA, i.e., initial appearance of the rostral NA followed by organization throughout the caudal extent followed by maturation of motoneurons, has also been noted in a histological study of the developing human NA (Brown, 1990). Further, the observed maturation of motoneurons in NA coincides with critical periods in the development of muscle fibers (Rubinstein and Kelly, 1981), and, as such, indicates the presence of coordinated developmental sequences between motoneurons and their target muscles (Ross et.al., 1987).

Motoneuron cell death (MCD) was quantitatively assessed in NA from the time the nucleus first appeared distinct, i.e., E17. The results of motoneuron counts demonstrated for the first time, that natural cell death does occur in the developing NA. Further, the

cell loss occurred entirely prenatally with no significant loss of cells observed between P1 and adults. While MCD has been shown to occur prenatally in most animals studied (see reviews, Grinnell and Herrera, 1981; Kuno, 1990), several studies in rodents have indicated an additional period of motoneuron death *postnatally* (Tada et.al., 1979; Rootman et.al., 1981; Scarisbrick et.al., 1990). These studies, however, employed neural tracing techniques and did not count motoneurons directly. Studies which have used standard counting methodologies to investigate the occurrence of cell death in rodents, with one exception, have found no postnatal loss of cells (Lance-Jones, 1982; Oppenheim, 1986; Oppenheim et.al., 1986; Friedland et.al., 1991a, 1991b; Friedland et.al., 1993). The finding of postnatal motoneuron death in a direct counting study by Nurcombe and co-workers (1981) may be related to the use of the Wistar strain of rat, as a similar study of sensory nuclei in the same strain also demonstrated some postnatal neuronal death (Ashwell and Waite, 1991). In contrast, this study, and those which indicate no postnatal cell death, counted cells in Sprague-Dawley rats (Oppenheim, 1986; Friedland et.al., 1991a, 1991b; Friedland et.al., 1992a, 1992c, 1992d). That a postnatal period of cell death was missed in this investigation was precluded by the choice of ages studied, i.e., within three hours of birth and six months postnatal. Further, the choice of six months as the adult age, was chosen to investigate specifically developmental cell death, rather than an aging process, as aging degeneration in NA occurs well after this time (Sturrock, 1990).

The quantitative assessment of MCD began at E17, however, observations of degenerating cells in NA indicated that MCD probably started just prior to E16. In addition, MCD was complete by P1, with little, if any cell death occurring on the last day of gestation. This temporal pattern of MCD corresponds well to other studies in the rat. Oppenheim (1986), using only one gestational age demonstrated cell death in the spinal cord just prior to E15 and ending by P1. A closer examination of prenatal stages by Harris and McCaig (1984) showed MCD between E15 and E21 in the brachial cord.

Studies on the mouse, staged to correspond to rats (Butler and Juurlink, 1992), indicated MCD in the spinal cord between E14.5 and E20.5 (Lance-Jones, 1982; Oppenheim et.al., 1986). Further, data on the mouse facial nucleus indicated the majority of MCD in the rat brainstem would occur later in gestation, around E16.5 through E20.5 (Ashwell and Watson, 1983). Brainstem studies in the chick, correlated with rat developmental stages (Butler and Juurlink, 1992), also demonstrated a later onset of MCD, E16 in the trigeminal motor nucleus (Arens and Straznicky, 1986) and E17 in the trochlear nucleus (Cowan and Wenger, 1967). As will be discussed in the next chapter, studies in the rat hypoglossal nucleus have demonstrated MCD largely between E15 and E19 (Friedland et.al., 1992a, 1992c, 1992d; Friedland et.al., 1993). These data indicate a caudal to rostral gradient in the onset, and possibly termination, of MCD in the rat. Specifically, onset of MCD in the spinal cord is around E14/E15; in XII, around E15; in NA, around E16; in the facial nucleus, around E16.5; in the trigeminal nucleus, around E16; and in the trochlear nucleus, around E17.

MCD in NA, between E17 and postnatal ages, accounted for an approximately 50% loss of motoneurons. That no degenerating cells were found at E15, few at E16, and many at E17, indicates that a small amount of cell death would have occurred prior to E17. Based on the rate of cell death at later stages and the prevalence of degenerating neurons at E17, total cell death in NA is likely to lie between 55 and 60%. This is not an unusual amount of MCD, as studies have reported from 20% to 80% cell death various in neuronal systems (see review, Oppenheim, 1991). Studies of MCD in the rat have found between 40% and 50% cell death in the spinal cord (Nurcombe et.al., 1981; Oppenheim, 1986). This is in accordance with studies of the chick in which similar amounts of cell death were observed (Hamburger, 1975; Chu-Wang and Oppenheim, 1978b; Laing, 1982). In contrast, the spinal cord of the mouse has shown between 60% and 70% motoneuron loss (Lance-Jones, 1982; Oppenheim, et.al., 1986). Brainstem studies in the mouse (facial nucleus) noted similar neuronal losses amounting to 68% cell death

(Ashwell and Watson, 1983). Brainstem motor nuclei in the chick and duck also exhibited motoneuron death but to a lesser degree: 49% motoneuron loss in the trigeminal nucleus (Arens and Straznicky, 1986) and 50% loss in the trochlear nucleus (Cowan and Wenger, 1967; Sohal, 1976). Thus, MCD in the rat NA appears to be greater than that in the rat spinal cord, but average when compared to brainstem studies across species. The only direct comparison in the rat brainstem is with the hypoglossal nucleus which exhibited considerably less MCD than that found in NA (Friedland et.al., 1992a, 1992c, 1992d; Friedland et.al., 1993; also see chapter 7). The various amounts of motoneuron death found between species and neuronal populations may reflect differences in postnatal demands on the innervated muscles or neuromuscular system. In addition, the course of MCD may reflect differences in the type and amount of afferent neuronal input to the motoneurons. Thus, MCD in NA may be larger than that in the rat spinal cord because of the complexity of URT control and because of the many respiratory, swallowing, and vocalizing centers which project to NA motoneurons.

The course of MCD is depicted graphically in figure 6.16. A slight decrease in cell number between E17 and E18 was statistically insignificant by two-tailed Student's t-test. That some cell death occurred prior to E17 (see discussion above), indicates that the period between E17 and E18 may represent a slight "plateau" in the occurrence of MCD. In addition, that fewer degenerating cells were apparent at E18 than at E17 further supports the possibility that cell death is reduced during this period. After E18 there was a dramatic reduction in motoneuron numbers through E20. Indeed, during this two day period over 70% of all observed motoneuron death occurred. Investigation of the E19 and E20 NA showed many degenerating neurons, more than at E18. There was a small loss of cells between E20 and P1 representing the "tail-end" of MCD. Few, if any, degenerating cells were seen in the P1 NA indicating the cessation of MCD by birth. These results indicate that MCD in NA may follow a biphasic course with cell death occurring prior to E17, slowing between E17 and E18, and resuming up until birth.

Studies on the tammar (*Macropus eugenii*), an Australian marsupial, have also observed a biphasic course of MCD (Comans et.al., 1987). Further, the course of cell death in the rat XII exhibited a small plateau shortly after the onset of MCD (Friedland et.al., 1992c, 1992d; Friedland et.al., 1993; see chapter 7). Similarly, in the human XII, two separate periods were found in which degenerating neurons were present (Nara et.al., 1989). Such a biphasic occurrence of MCD may be exaggerated in some rodent strains and account for the reports of a second, postnatal period of motoneuron death (Tada et.al., 1979; Rootman et.al., 1981; Scarisbrick et.al., 1990). Interestingly, Lance-Jones (1982) while finding no data to support earlier reports of postnatal motoneuron death in the mouse, did demonstrate two distinct periods of MCD entirely prenatally. A biphasic course of MCD may indicate differences in the nature of the epigenetic signals directing MCD at early and late developmental periods. For example, the initial phase of cell death may be in response to the peripheral target and refine neuromuscular synapses. The later phase may reflect afferent neuronal signals and serve to establish specific connections ensuring proper postnatal neuromuscular control.

While the course of MCD was defined by changes in the NA cell population, data at each age provided an empirical estimate of total motoneuron numbers (table 6.2). This data indicated a population of approximately 1400 motoneurons in the adult rat NA. No studies were found with which to compare directly these results, however, Lawn (1966) reported approximately 1500 motoneurons in the rabbit NA. The analysis of motoneuron counts with respect to litter showed no appreciable differences in motoneuron numbers between offspring of different mothers. This is not surprising as all rats were of the same strain and essentially express clonal genotypes. Analysis of differences between the left and right NA showed no laterality at any stage. Thus, experiments utilizing unilateral nerve transections and target manipulations (e.g., Pollack, 1969; Hollyday and Hamburger, 1976; Oppenheim, et.al., 1978; Yu, 1988) appear valid in using the unoperated side as a control for the comparison of motoneuron numbers.

These data have demonstrated the occurrence of MCD in the rat NA during late gestation. As such, late gestation represents a period of dramatic change in the motoneuron population innervating structures of the URT. During this period, the course of MCD is responsive to epigenetic signals from laryngeal and pharyngeal muscles, and from afferent neurons of the brain and brainstem. This coordinated developmental sequence, in large part, establishes the neuromuscular specificity necessary for proper postnatal URT control. Thus, MCD represents a critical period in the formation of URT neuromuscular control.

Chapter 7

Hypoglossal Nucleus: Motoneuron Cell Death

7.1 Introduction

This study investigates the development of the hypoglossal nucleus (XII). This nucleus contains motoneurons which innervate muscles of the tongue (see chapter 2), the principle structure of the oral cavity and anterior pharynx. As such, XII is intimately involved in the control and coordination of many aerodigestive and vocal functions. This study seeks to characterize the patterns of MCD in the hypoglossal nucleus and to define critical periods in the establishment of URT neuromuscular control.

7.2 Experimental Design

7.2.1 Motoneuron Counts in XII

The hypoglossal nucleus (XII) appears as a well-defined prominent nucleus in prenatal brainstems. Additionally, it is clearly localized along the fourth ventricle and is in intimate contact with one other nucleus, the dorsal motor nucleus of the vagus (dX). As dX is well-defined throughout its rostral-caudal extent, accidental inclusion of its parasympathetic neurons in counts of XII motoneurons was not a concern. Thus, unlike the study of NA, specific counting protocols designed to exclude extraneous non-URT motoneurons in the assessment of MCD played a small role in counting XII.

The only recognized source for the accidental inclusion of non-URT motoneurons in XII cell counts was in the rostral-most extent of the adult nucleus. In the adult brainstem, the perihypoglossal nucleus, which is just rostral to XII, stains strongly for Nissl and some of its most caudal neurons may be mistaken for the rostral-most XII motoneurons (Yu, personal communication). However, the number of cells in sections at this location was relatively small and it is estimated that few, if any, non-URT neurons could have been erroneously included in the adult cell counts.

The principal focus in counting XII was in ensuring the *inclusion* of all motoneurons innervating the tongue. This was a concern with regard to a small subset of hypoglossal

motoneurons located just ventrolateral to the well-defined body of the nucleus; motoneurons to the geniohyoid muscle (see chapter 2). In prenatal specimens, geniohyoid neuroblasts demonstrated the same prominent morphology as other URT motoneurons and were clearly distinguished from surrounding reticular neurons. Thus, inclusion of these motoneurons in the cell counts required no more than recognition of their location and awareness of their existence. These neurons were also readily evident in Nissl stained postnatal material thus preventing their possible exclusion from MCD assessment in XII.

7.2.2 Materials

Development of XII was investigated from E13 through E19, with counts made of hypoglossal motoneurons at E16, E17, E18, E19, P1 and adult. As in the investigation of NA, MCD was assessed from the earliest age at which XII was fully distinct and successively thereafter until results showed a cessation of motoneuron death. Additionally, as in NA, 10 animals per age were used representing three different litters with no litter represented by less than three specimens. Weights and crown-rump lengths were taken of prenatal specimens used for assessment of MCD and are shown in table 7.1 and graphically in figure 7.1. While many specimens were also used for the study of cell death in NA, due to difficulties in fixation (see chapter 5) several specimens were unique to this study.

7.3 Results

This investigation has provided insight into the development of the brainstem and the formation and maturation of XII. Overall brainstem development was discussed in chapter 6 and will not be presented here. Rather, this section will focus on results specific for XII, in particular: 1) histological observations on the differentiation of XII

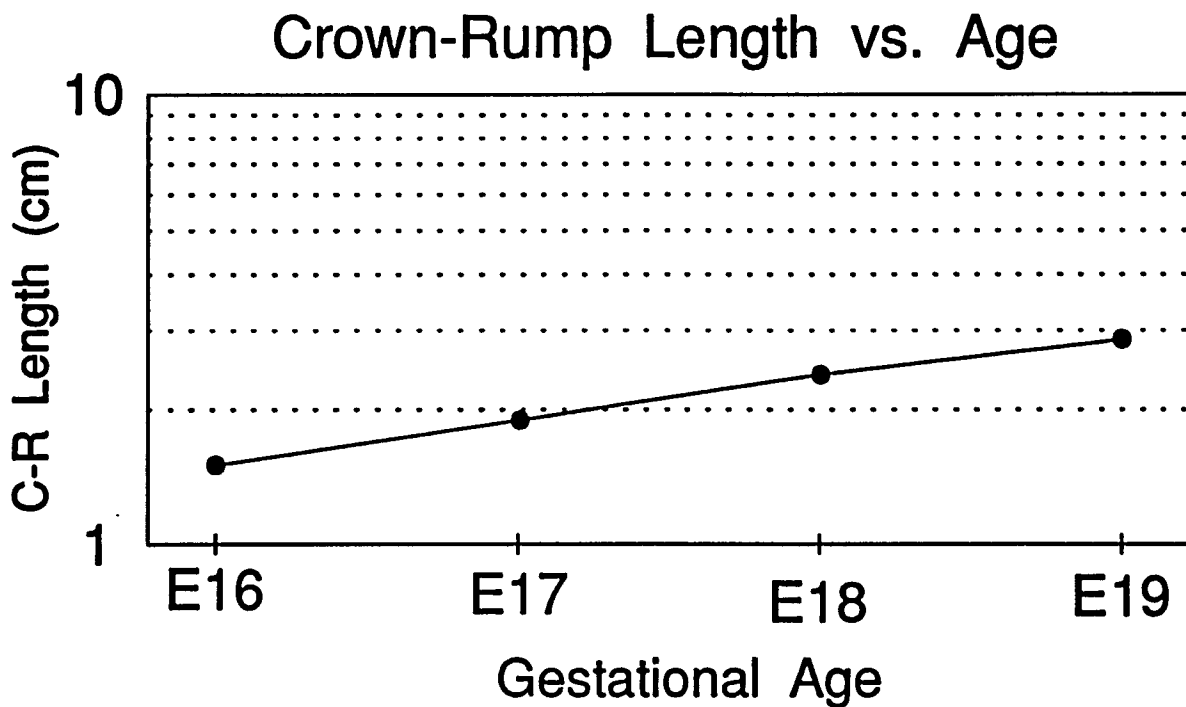
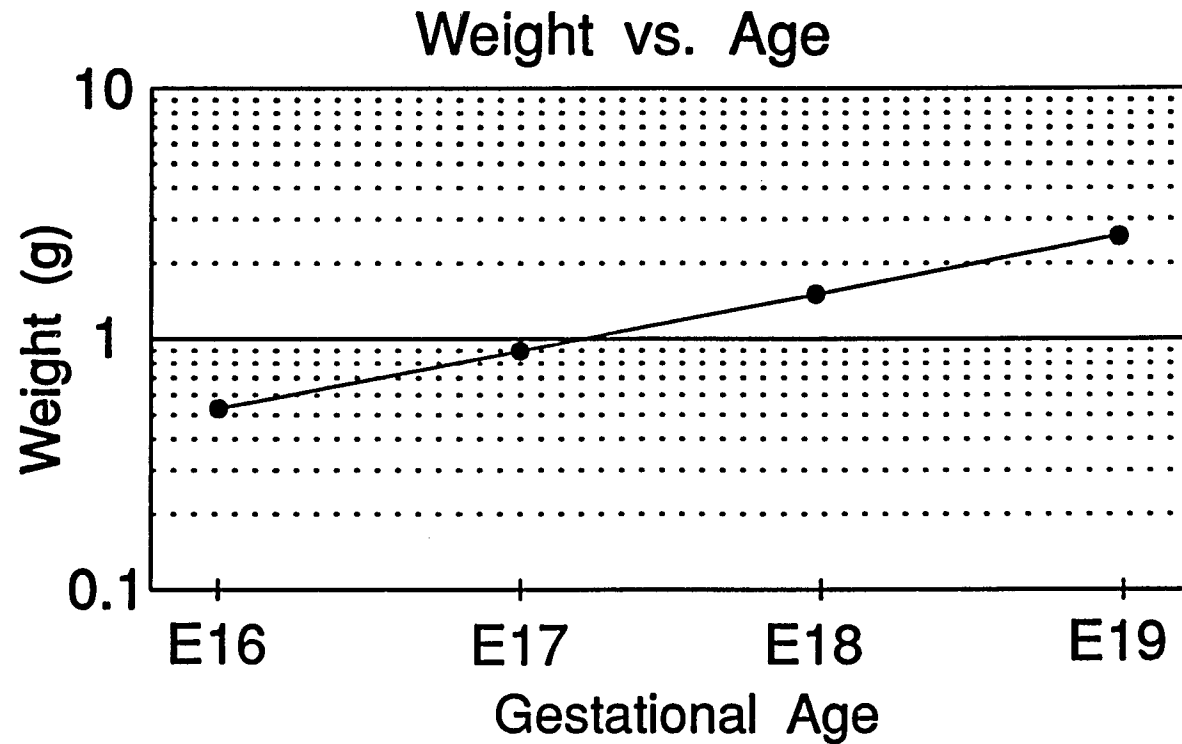
Table 7.1: Weights and Crown-Rump Lengths of Rats Used For the Assessment of MCD in XII

E16	Weight (g)	C-R Length (cm)	E19	Weight (g)	C-R Length (cm)
E16-A2	.6095	---	E19-A4	2.3421	---
E16-A4	.5965	---	E19-A5	2.3691	---
E16-A6	.6081	---	E19-A6	2.3047	---
E16-B1	.4651	1.499	E19-B2	2.6499	---
E16-B3	.5148	1.545	E19-B3	2.6876	---
E16-B5	.5163	1.535	E19-B4	2.7147	---
E16-B6	.5172	1.512	E19-B5	2.6509	---
E16-C1	.5623	1.530	E19-C1	2.8569	2.955
E16-C3	.4365	1.410	E19-C3	2.6986	2.842
E16-C5	.4585	1.462	E19-C5	2.5607	2.789
Mean	.5285	1.499	Mean	2.5835	2.862
SEM	.0212	.018	SEM	.0616	.049
E17	Weight (g)	C-R Length (cm)	P1	Weight (g)	C-R Length (cm)
E17-A3	1.0095	---	P1-E1	5.912	---
E17-A4	.9404	---	P1-E2	6.1878	---
E17-A5	.9118	---	P1-E3	6.9665	---
E17-B3	.9401	---	P1-F1	8.1955	---
E17-B4	.8970	---	P1-F2	8.6615	---
E17-B5	.9159	---	P1-F3	7.015	---
E17-B6	.8900	---	P1-F4	7.7683	---
E17-C2	.8193	1.912	P1-G5	5.7836	---
E17-C3	.7778	1.879	P1-G6	6.5673	---
E17-C5	.8195	1.872	P1-G7	6.7715	---
Mean	.8921	1.888	Mean	6.9829	n/a
SEM	.0231	.012	SEM	.3209	n/a
E18	Weight (g)	C-R Length (cm)	Adult	Weight (g)	C-R Length (cm)
E18-D2	1.3005	---	A-8	600	---
E18-D3	1.5355	---	A-9	685	---
E18-D4	1.3091	---	A-10	635	---
E18-D5	1.1715	---	A-11	645	---
E18-E4	1.6304	2.346	A-12	735	---
E18-E5	1.6167	2.353	A-13	443	---
E18-E6	1.8548	2.305	A-15	426	---
E18-F3	1.5584	2.505	A-16	358	---
E18-F4	1.7632	2.492	A-17	354	---
E18-F5	1.4459	2.341	A-18	354	---
Mean	1.5086	2.390	Mean	523.5	n/a
SEM	.0663	.035	SEM	50.27	n/a

(n/a = not applicable)

Figure 7.1

Weight and Crown-Rump Length Versus Age in Rats Used to Assess MCD in XII



and the generation of general somatic motoneurons; and 2) maturational changes within XII, including the occurrence of MCD. These results are shown in figures 7.2-7.14 and table 7.2.

7.3.1 Hypoglossal Nucleus Formation

The mature XII is found in the dorsomedial brainstem along the fourth ventricle. Studies of this region in the E13 rat failed to observe clusters of neuroblasts that could be considered XII although hypoglossal nerve fibers were seen exiting the brainstem ventrally at this level (figure 7.2). At this level, a large "pool" of neuroblasts was observed at the basal plate but could not be identified as hypoglossal motoneurons (figure 7.3). By E14 a small and ill-defined XII was observed in the dorsomedial brainstem (figures 7.4 and 7.5). The precise boundaries of the nucleus, particularly at the ventrolateral border were difficult to determine. At E15, XII was readily apparent, present in all specimens and found in all serial sections through the nucleus (figure 7.6 and 7.8). The nucleus was densely packed with neurons which were found in several planes of focus even in the relatively thin 8µm sections (figure 7.8). By E16, XII was fully distinct and began to exhibit subnuclear organization as evidenced by the appearance of the small, ventrolateral, geniohyoid group of motoneurons (figures 7.7 and 7.9). As at E15, the nucleus was densely packed with neuroblasts but the increase in size of the region allowed easier assessment of individual neurons.

Once XII was fully formed, most changes appeared to be associated with organization of the motoneurons within XII. There was a decrease in the apparent density of hypoglossal motoneurons by E17 which continued through E19 (figures 7.10 and 7.11). At these stages the borders of XII were clearly defined by small cells except around the ventrolateral geniohyoid subdivision of motoneurons. Some sections at these ages also exhibited division of XII into dorsolateral and ventromedial groups (see chapter 2). Such division of the nucleus was inconsistently observed and, therefore, independent

assessment of protrusor and retrusor motoneurons was not possible. By E19, neuroblasts in XII began to exhibit adult-like morphology with an increase in cytoplasmic staining defining a classic "triangular" neuronal shape. Little change in neuronal morphology or nucleus organization was observed after E19. However, in both P1 and adult brainstems, XII was no longer defined by small, peripherally located cells (figures 7.12 and 7.13).

7.3.2 Motoneuron Cell Death

Results of counts made of motoneurons in XII in E16 through adult rats are shown in table 7.2, and graphically in figure 7.14. The nucleolar counts per section are listed in Appendix C. At E16 there were 5128 ± 18 (average $\pm$ SEM) motoneurons in XII. This was followed by a statistically significant decrease in motoneuron numbers through E17 where 4428 ± 211 motoneurons were found ($P \leq .001$). Motoneuron numbers were relatively stable through E18 where 4259 ± 101 motoneurons were counted. The difference in motoneuron counts at E17 and E18 were shown to be statistically insignificant by Student's t-test. A subsequent decrease in the hypoglossal motoneuron population was found between E18 and E19 where approximately 3500 cells were found ($P \leq .001$). Postnatally, motoneuron counts indicated 3330 ± 52 cells at P1 which were statistically insignificant from those at E19, as well as from motoneuron counts in the adult (3294 ± 100).

Histological investigation of XII for the occurrence of cell death showed infrequent degeneration in the E15 nucleus with an increase in the number of profiles by E16. Many dying cells were observed at E17 and E19, but were less prominent at E18. While fewer apoptotic cells were seen at E18, the nucleus had a "dirty" appearance with small, basophilic particles scattered in the neuropil. At E20, there were essentially no dying cells as degenerating profiles were seldomly seen. In the postnatal XII no degeneration was observed at P1 or in adults.

Figure 7.2: Transverse section through an E13 rat brainstem at the level of XII.
Although a distinct nucleus was not observed at this stage, the level of hypoglossal motoneurons was determined by observations of hypoglossal nerve rootlets emerging from the ventral brainstem (left nerve indicated by arrow). The fourth ventricle (v) is indicated for orientation as XII lies along the ventricle dorsomedially in the brainstem. For further orientation see figure 2.2 and chapter 4. (H&E, 80x)

Figure 7.3: Closer view of above E13 brainstem in the dorsomedial region where the mature XII will be found. As in the E13 brainstem at the level of NA, a thick proliferative zone (star) is observed as are numerous mitoses along the ventricle (e.g., small arrow). Near this region, a large pool of immature neuroblasts can be seen. These cells, however, do not form a distinct nucleus. This pool is indicated on the left side by the large arrow. (H&E, 200x)

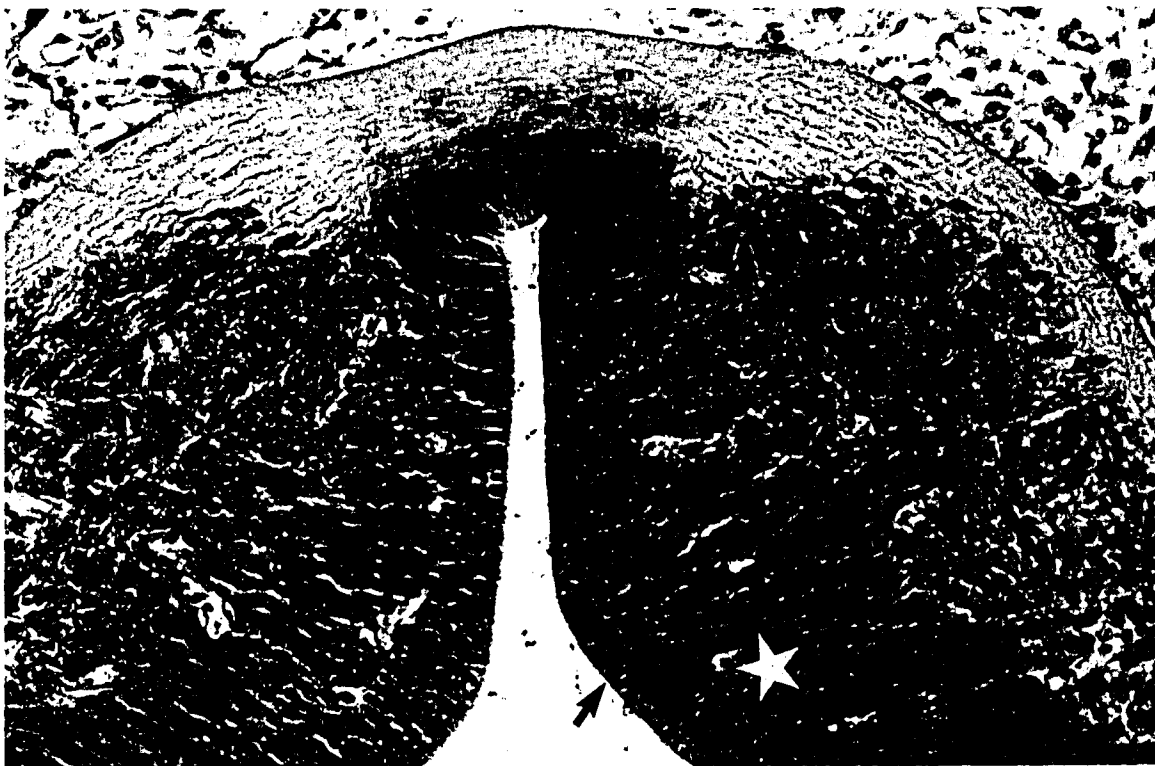


Figure 7.4: Transverse section through an E14 rat brainstem at the level of XII. This section is oriented in the same manner as figure 7.2. The brainstem level was similarly established by observations of the emerging hypoglossal nerve rootlets (indicated on left side by small arrow). In the dorsomedial brainstem, a diffuse cluster of cells could be seen defining the left and right XII (large arrow indicates nucleus on left side). (H&E, 40x)

Figure 7.5: Closer view of the dorsomedial region of the above E14 brainstem. At this stage a small cluster of neuroblasts was first seen defining a diffuse XII (arrows indicate left and right nucleus). This nucleus was not seen in all sections through the brainstem and cell counts were not taken at this stage. (H&E, 200x)



Figure 7.6: Transverse section through the left and right XII in an E15 rat brainstem. At this stage XII (left XII indicated by arrow) is more distinct and present in all specimens and serial sections. Although clearly seen at this stage, the borders of the nucleus were often diffuse along the ventrolateral side (e.g., note area near arrow). (H&E, 125x)

Figure 7.7: Transverse section through the left and right XII in an E16 rat brainstem (arrow indicates left XII). The nucleus at this stage is distinct and has increased in size relative to that seen at E15 (see above). The borders of the nucleus were sometimes defined by small cells as seen in this section in the right nucleus (e.g., small arrow). (H&E, 125x)

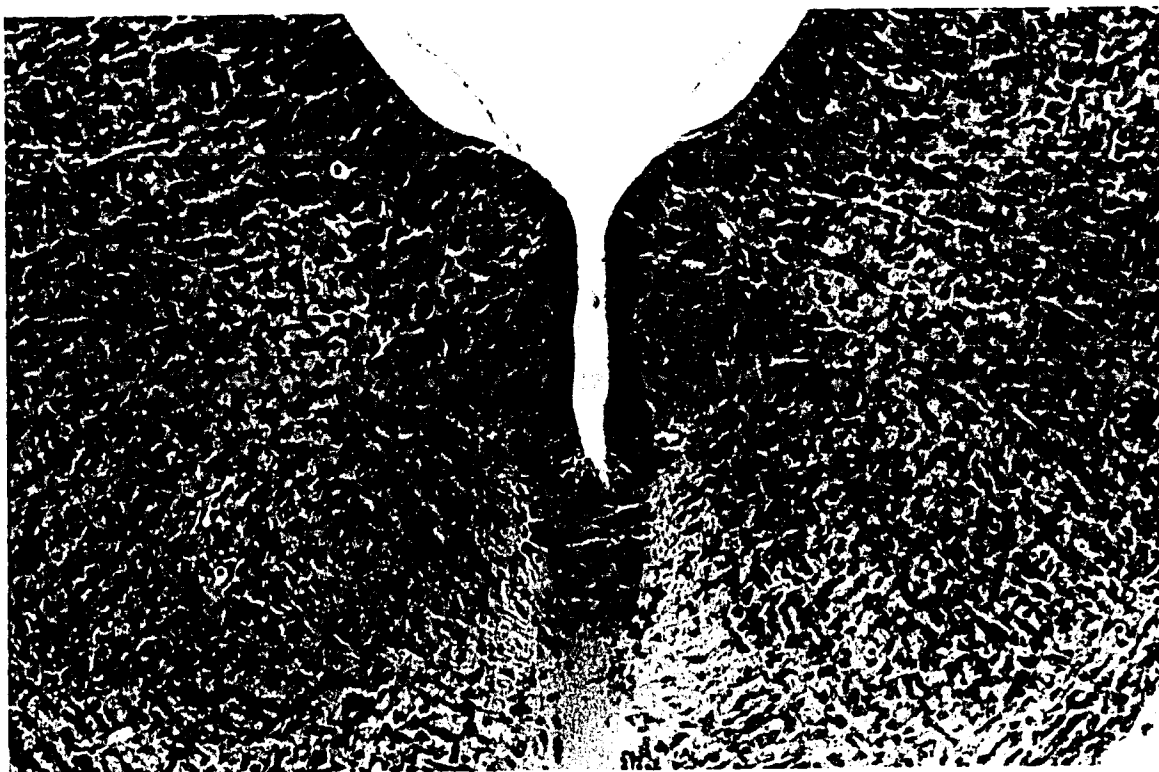


Figure 7.8: Transverse section through the left and right XII in an E15 rat brainstem. Although XII was apparent in all specimens, motoneuron counts were not obtained at this stage as the density of cells in the nucleus created several planes of focus making identification of individual cells and nucleoli unreliable. (H&E, 200x)

Figure 7.9: Transverse section through the left and right XII in an E16 rat brainstem. The nucleus was densely packed with cells at this stage but less so than at E15 (see above) thus enabling counts of motoneurons to be taken. Counts at this stage indicated approximately 5100 motoneurons in each side. See text for further details on cell counts. (H&E, 200x)



Figure 7.10: Transverse section through the left and right XII in an E17 rat brainstem. By this stage XII had increased considerably in size and the density of cells appeared to decrease. Counts at this stage showed about 4400 motoneurons indicated the occurrence of motoneuron cell death during this period (see text). (H&E, 200x)

Figure 7.11: Transverse section through the left and right XII in an E19 rat brainstem. There was a further increase in size and further loss of motoneurons in XII through E19. Counts at this stage indicated the tail-end of motoneuron cell death and indicated approximately 3500 cells in XII (see text for further discussion). (H&E, 200x)

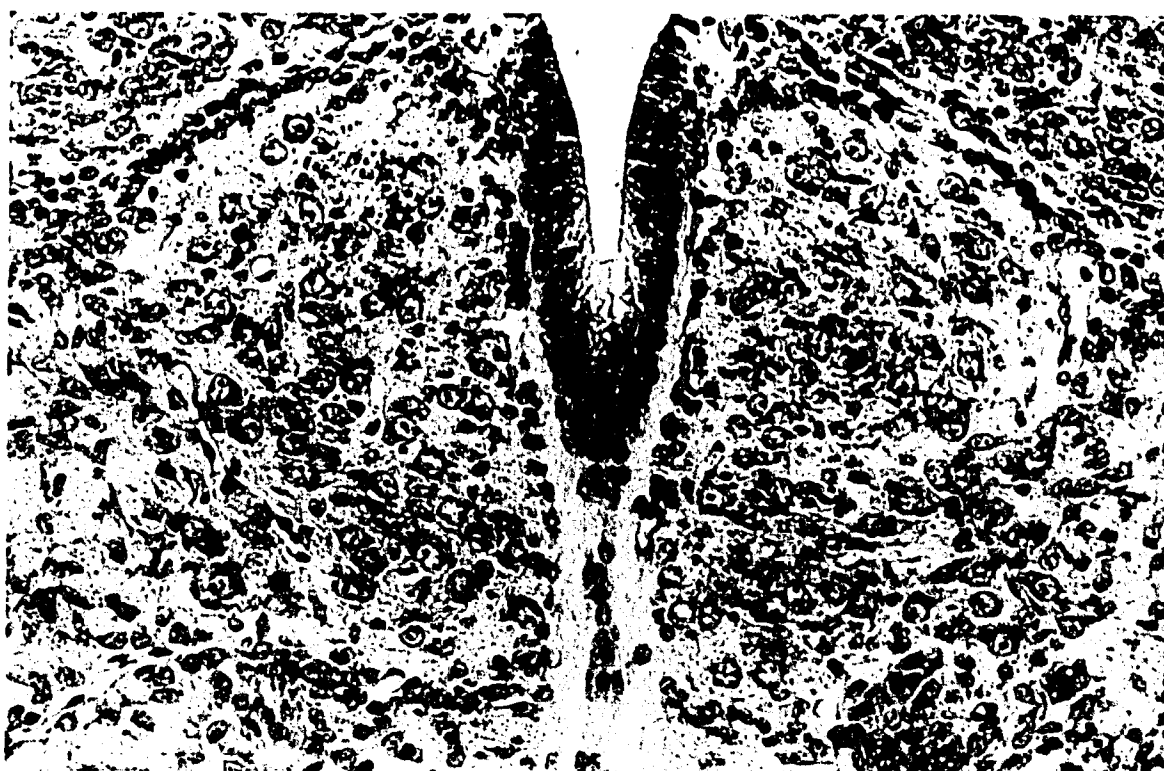
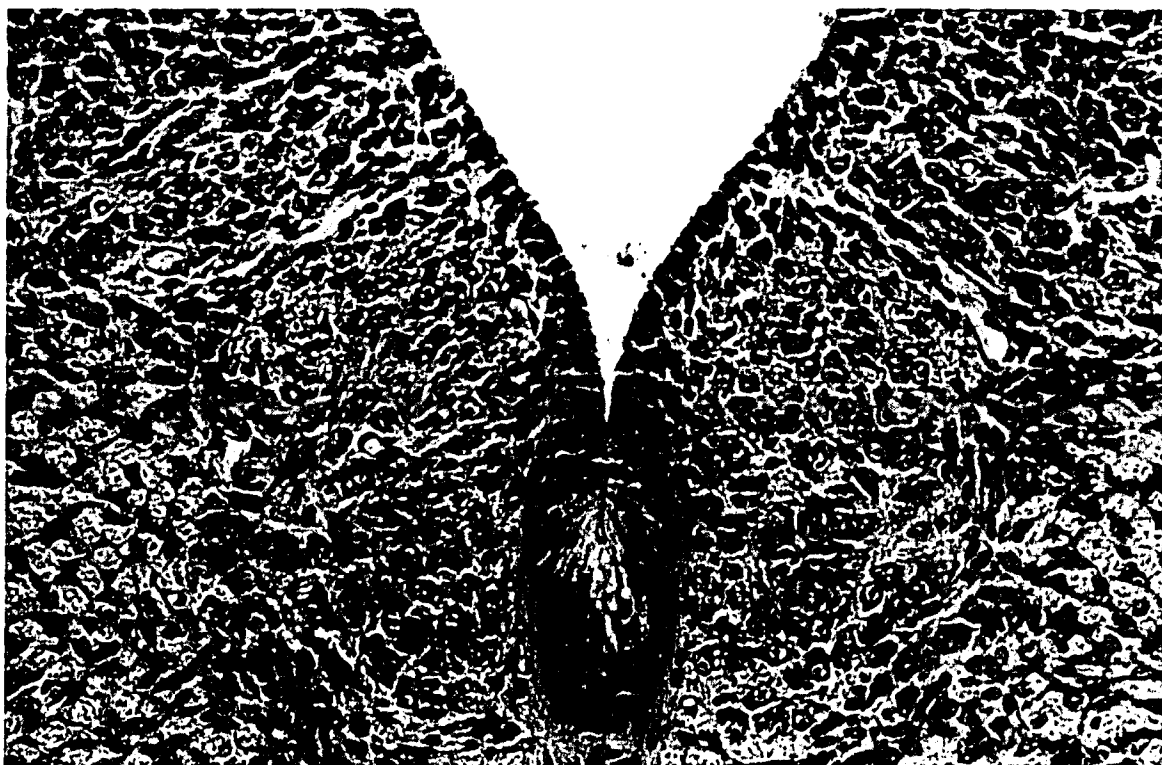


Figure 7.12: Transverse section through the left and right XII in a P1 rat brainstem. The precise boundaries of XII are difficult to indicate in this figure as the nuclei encompass virtually the entire frame. The arrow indicates the inferior border of the left XII. Large motoneurons with distinct nucleoli can be seen throughout the nucleus. Motoneuron counts at this stage established the postnatal population of motoneurons at about 3300. (cresylviolet, 125x)

Figure 7.13: Transverse section through the left and right XII in an adult rat brainstem. The nuclei extend past the lateral borders of this figure but the superior borders can be seen and is indicated for the left XII (arrow). Above the arrow are the smaller parasympathetic motoneurons of the dorsal motor nucleus of the vagus. Counts at this stage were similar to those at P1 and indicated no postnatal loss of motoneurons in XII. (cresylviolet, 125)

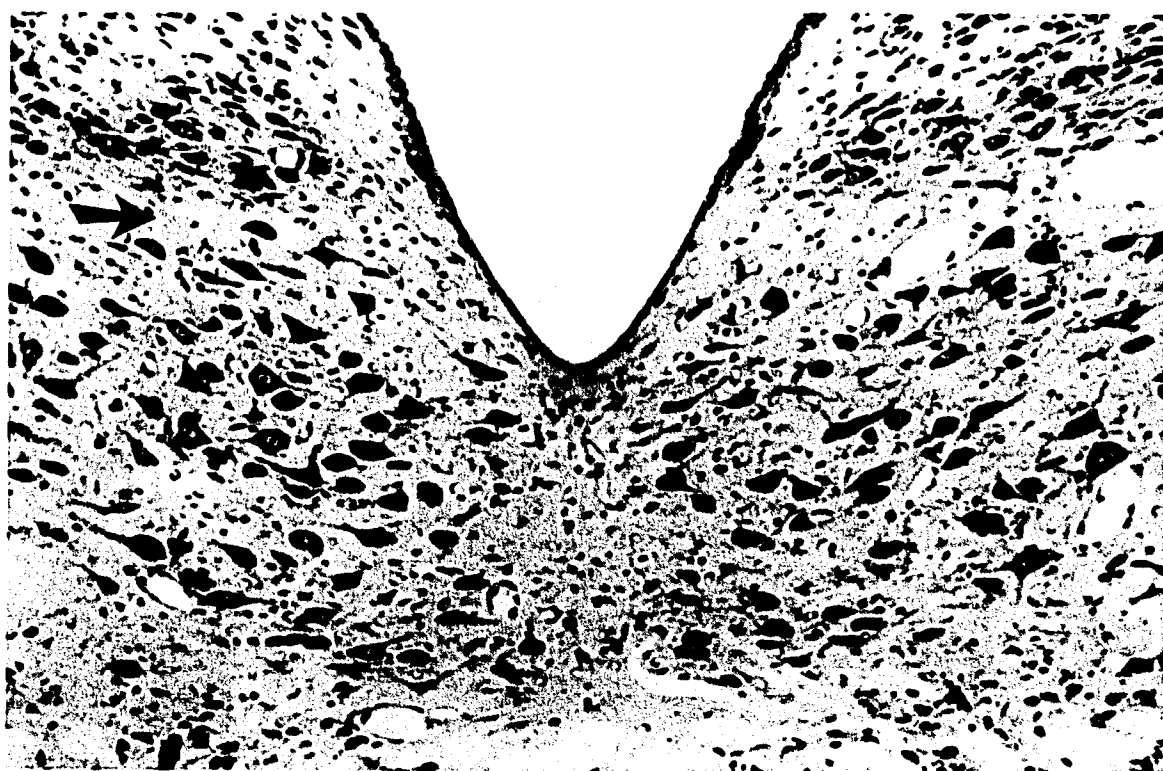


Table 7.2: Estimated Total Cells in the Hypoglossal Nucleus

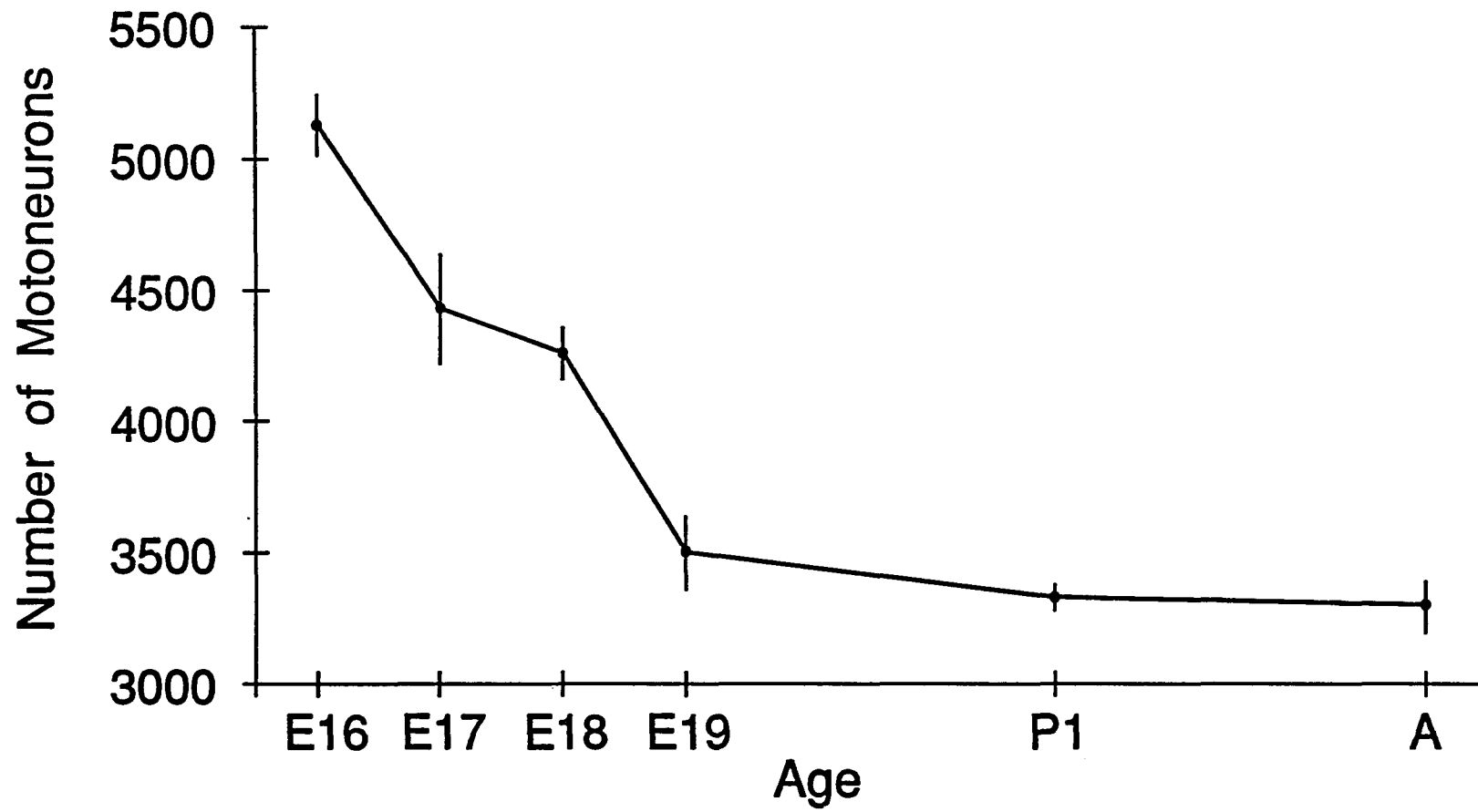
E16	Left	Right	Average	E19	Left	Right	Average
E16-A2	5210	4745	4977.5	E19-A4	3530	3245	3387.5
E16-A4	4655	4785	4720	E19-A5	3250	3165	3207.5
E16-A6	5070	5105	5087.5	E19-A6	3865	3830	3847.5
E16-B1	5355	5230	5292.5	E19-B2	3220	3310	3265
E16-B3	5485	5375	5430	E19-B3	4085	3865	3975
E16-B5	5310	5530	5420	E19-B4	2755	2555	2655
E16-B6	6000	5345	5672.5	E19-B5	3275	3290	3282.5
E16-C1	4525	4425	4475	E19-C1	3740	3915	3827.5
E16-C3	5075	5170	5122.5	E19-C3	3930	3995	3962.5
E16-C5	5185	4980	5082.5	E19-C5	3680	3470	3575
Mean	5187	5069	5128	Mean	3533	3464	3498.5
SEM	137.94	112.66	117.55	SEM	135.49	148.98	140.01

E17	Left	Right	Average	P1	Left	Right	Average
E17-A3	4750	4835	4792.5	P1-E1	3490	3530	3510
E17-A4	5535	5570	5552.5	P1-E2	3475	3105	3290
E17-A5	3515	3655	3585	P1-E3	3770	3350	3560
E17-B3	4205	4265	4235	P1-F1	3070	2930	3000
E17-B4	4755	5110	4932.5	P1-F2	3340	3295	3317.5
E17-B5	4685	4430	4557.5	P1-F3	3375	3025	3200
E17-B6	3675	3390	3532.5	P1-F4	3380	3335	3357.5
E17-C2	4965	4720	4842.5	P1-G5	3360	3290	3325
E17-C3	4120	4390	4255	P1-G6	3360	3435	3397.5
E17-C5	4065	3925	3995	P1-G7	3380	3300	3340
Mean	4427	4429	4428	Mean	3400	3259.5	3329.75
SEM	206.90	221.56	211.04	SEM	57.49	61.78	51.91

E18	Left	Right	Average	Adult	Left	Right	Average
E18-D2	4375	4415	4395	A-8	3645	3507	3576
E18-D3	4025	4105	4065	A-9	3294	3135	3214.5
E18-D4	3875	3830	3852.5	A-10	3084	3111	3097.5
E18-D5	4550	4145	4347.5	A-11	3072	3162	3117
E18-E4	4165	4285	4225	A-12	2907	2976	2941.5
E18-E5	4380	4705	4542.5	A-13	3564	3483	3523.5
E18-E6	3500	3845	3672.5	A-15	3345	3210	3277.5
E18-F3	4525	4550	4537.5	A-16	3840	3945	3892.5
E18-F4	4585	4405	4495	A-17	2985	2934	2959.5
E18-F5	4415	4495	4455	A-18	3357	3333	3345
Mean	4239.5	4278	4258.75	Mean	3309.3	3279.6	3294.45
SEM	116.40	97.76	101.01	SEM	101.66	100.51	99.73

Figure 7.14

Estimated Number of Motoneurons Versus Age in XII:
Graphical Depiction of the Course of MCD



7.3 Discussion

The tongue is the principal muscular structure of the oral cavity and, as such, plays a large part in establishing patterns of respiration, deglutition, and vocalization. While these activities are crucial to the maintenance of normal life, little is known of the development of the neuronal centers controlling the tongue. The hypoglossal nucleus (XII) contains general somatic motoneurons which project to the tongue. This study has investigated the development of XII in pre- and postnatal rats in an effort to define critical periods in the establishment of upper respiratory tract (URT) neuromuscular control. Particular attention has been paid to the formation of the nucleus and the subsequent maturation of its motoneurons, specifically the occurrence of motoneuron cell death.

Histological assessment of neurogenesis, as defined by the presence of ventricular layer mitoses, was discussed in the previous chapter. The results showed many mitoses along the fourth ventricle at E13 and E14 thus indicating the occurrence of neurogenesis at this time. While these results conformed with a previous report on the timing of generation of ambigular motoneurons (Altman and Bayer, 1980), studies on the genesis of hypoglossal motoneurons have demonstrated a peak production at E10 and E11 (Altman and Bayer, 1980; Phelps et.al., 1990). Thus, the observed "pool" of neuroblasts in the E13 brainstem (figure 7.3) may contain all the hypoglossal neurons generated at earlier stages. These neuroblasts may not form a discernible nucleus, however, until E14, when XII was first found (figure 7.5). The initial appearance of XII at E14 was one day prior to the first observation of NA, and prior to the dramatic changes in brainstem size and organization noted at E15 (see previous chapter). This "precocious" development of XII, relative to NA, may be the result of earlier generation of hypoglossal motoneurons. Alternatively, this difference in timing may be due to the need for cells to migrate to NA, while hypoglossal motoneurons are generated in the dorsomedial brainstem at their mature location. As such, while cells migrate out of the proliferative zone at E13 and

E14, hypoglossal motoneurons would remain stationary and thus appear in the mature location of XII before ambigular neurons arrived in the ventrolateral brainstem.

After its initial appearance at E14, XII became more distinct through E16. Over this two day period, motoneurons within XII appeared to become more organized and the ventrolateral subdivision associated with geniohyoid motoneurons emerged. In addition, while the nucleus remained dense with neurons, individual cells were easier to discern as they spread throughout XII. This was in contrast to the E15 XII in which neurons frequently overlapped one another and individual cells, particularly nuclei, were difficult to distinguish. For this reason, quantitative analysis of MCD was not initiated until E16 so that reliable cell counts could be obtained. The temporal pattern of nucleus organization in XII appeared similar to that for NA. That is, both nuclei appeared fully distinct two days after they were first observed in their adult location. However, the precocious development of XII relative to NA continued as it appeared fully distinct at E16 while NA was not consistently observed until E17. This temporal pattern was also seen in the maturation of the motoneurons as cells in XII first began to exhibit adult-like morphologies at E18 while those in NA showed mature morphologies at E19.

Further investigation of the maturation of XII focused on the occurrence of motoneuron cell death (MCD). These results, for the first time, have precisely described the course of normal neuronal degeneration in the motor nucleus to the tongue. That cell death was, in fact, observed is consistent with the recent findings of dying cells in the developing human XII (Nara et.al., 1989). While this study found degenerating neurons in XII at several stages of prenatal human development, however, their motoneuron counts failed to define a precise period of hypoglossal MCD. In contrast, a study by Milutinovic and co-workers (1992) demonstrated the loss of over 80% of the initial population of motoneurons in the human XII between 12.5 weeks of gestation and birth. These results, however, must be viewed cautiously as their method of counting was not

by standard methods, they had a very small sample size, and the amount of observed cell death was greater than in any motor nucleus thus far studied.

The results of this study have demonstrated, not only that MCD is found in XII, but the developmental period during which this process occurs. Examination of XII for degenerating neurons indicated that MCD begins just prior to E15. This was apparent by the lack of degenerating profiles in XII at E14 and the appearance of dying cells at E15. Motoneuron counts were initiated at E16 and demonstrated a loss of cells through E19 with no further degeneration thereafter. Thus, as in NA, MCD in the rat XII occurred entirely prenatally. As described in the previous chapter, the presence of an additional period of cell death in the rodent nervous system *postnatally* is controversial with several studies showing a significant loss of cells (Tada et.al., 1979; Rootman et.al., 1981; Scarisbrick et.al., 1990) and others demonstrating no postnatal degeneration (Lance-Jones, 1982; Oppenheim, 1986; Oppenheim et.al., 1986). This study provides evidence from two motor nuclei, NA and XII, that no postnatal cell death occurs in the Sprague-Dawley strain of rat.

The timing of the onset of MCD in various nuclei and species was discussed in chapter 6. These studies indicated that cell death in the rat spinal cord began around E15 (Harris and McCaig, 1984; Oppenheim, 1986) which is consistent with the present findings in XII. However, studies of upper brainstem motor nuclei such as the trochlear, trigeminal and facial, indicated an onset of MCD at later stages of development (Cowan and Wenger, 1967; Ashwell and Watson, 1983; Arens and Straznicky, 1986). Comparing data between XII and NA showed MCD in XII to initiate approximately one gestational day prior to that in NA. This is consistent with the precocious development of XII relative to NA seen in nucleus formation, organization, and motoneuron maturation. These differences in timing of MCD between spinal cord, lower brainstem, and upper brainstem strongly indicate a caudal-rostral temporal gradient of neuronal maturation (Friedland et.al., 1992c. 1992d).

The difference in timing of maturation between XII and NA was also evident in the assessment of the termination of MCD. While MCD in NA continued up until birth, cell death in XII ended considerably earlier, at E19. Although there was a slight decrease in motoneuron numbers between E19 and P1 the difference was not statistically significant. Further, examination of the E20 XII showed few if any degenerating motoneurons indicating the cessation of MCD at least two days prior to birth. Comparison with other nuclei and species (correlated with rat developmental stages) showed that cell death in spinal cord and higher brainstem motor nuclei typically continued through E20 to end at E21 (Lance-Jones, 1982; Harris and McCaig, 1984; Ashwell and Watson, 1983; Friedland et.al., 1992a). Thus, the hypoglossal motor pool is fully established prior to other motor nuclei and significantly before birth. This may reflect functional demands placed on the tongue neuromuscular system immediately postnatal for suckling and thus survival of the organism.

Motoneuron counts between E16 and P1 demonstrated a loss of approximately 35% of cells during MCD. The large number of degenerating cells at E16 indicated additional cell death prior to this time. Based upon the rate of cell death at later stages, compared to the number of degenerating profiles, total cell death in XII is probably between 40 and 45%. This is consistent with most findings of cell death in the rat (Nurcombe et.al., 1981; Oppenheim, 1986; Ashwell and Waite, 1991) and, interestingly, also similar to MCD in the chick (Cowan and Wenger, 1967; Hamburger, 1975; Chu-Wang and Oppenheim, 1978b; Laing, 1982; Arens and Straznicky, 1986). It is, however, less than the average of about 65% cell death seen in the mouse (Lance-Jones, 1982; Ashwell and Watson, 1983; Oppenheim et.al., 1986). It would be interesting to see whether differences in brain size between closely related species (i.e., rats and mice) could be attributable, in part, to differences in the amount of prenatal cell death.

The course of MCD in the rat XII is depicted graphically in figure 7.14. While there were significant decreases in cell numbers between E16 and E17, and again between E18

and E19, no statistically significant change was observed between E17 and E18. This decrease in the amount of cell death corresponded to a decrease in observed degenerating motoneurons in XII at E18. This "plateau" in the course of MCD was also seen in NA at the same developmental stage. As discussed in the previous chapter, several studies have indicated a biphasic course of MCD in mammals (Lance-Jones, 1982; Comans et.al., 1987; Friedland, 1993). Further, as indicated, these phases may represent differences in the epigenetic signals to which motoneurons respond during early and late stages of MCD. Interestingly, this plateau occurred between E17 and E18 in both NA and XII. As such, it is the only developmental phenomena which did not exhibit temporal variation. That is, all other maturation events occurred at least one day earlier in XII except for this relative stasis in the course of MCD. This indicates that a global change in the organism may occur at this time, universally affecting both, perhaps all, nuclei, and overriding local intercellular signals. While these changes may involve shifts in the morphology or activity of the prenatal URT, or fluctuations in hormone levels, further investigation is needed to explain fully the factors leading to a temporary stasis in the course of MCD.

While this study has primarily focused on the changes in motoneuron numbers between developmental stages, counts at each age have provided an empirical estimate of total motoneuron numbers (figure 7.2). These results have indicated slightly more than 3300 motoneurons in the adult XII. These results are similar to the findings of Yu (1988) and Lewis and co-workers (1971) who have demonstrated approximately 3500 motoneurons in the rat XII. In addition, this study showed no differences between hypoglossal motoneuron populations in males or females at neonatal or adult stages. This is in agreement with the findings of Yu (1988) and indicates that although androgens may play a significant role in motoneuron regeneration, they have little effect on the normal course of development of XII.

Interestingly, between the rat URT motor nuclei there was a considerable difference in the amount of cell death observed, 40-45% in XII versus 55-60% in NA. NA may undergo more cell death because of the relative complexity of the organization of its motoneurons. For example, NA is organized into many subnuclei, corresponding to glossopharyngeal and vagal nerves, and to palatal, pharyngeal, laryngeal and esophageal motoneurons. Errors in contacts with muscles, and with afferent neurons may be numerous and more cell death would be necessary to refine these contacts. Hypoglossal motoneurons are largely arranged into only two groups: a dorsolateral retrusor group and a ventrolateral protrusor group. Thus, contacts between muscles and neurons may be simpler and less cell death would be necessary to ensure neuromuscular specificity.

This study has demonstrated the occurrence of MCD during late gestation in the rat XII. The observed cell death in XII largely coincides with naturally occurring neuronal degeneration in NA, and defines E16 through birth as a dynamic period in the development of URT motor nuclei. During this period, hypoglossal and ambigular motoneurons are responsive to epigenetic signals from the tongue, larynx and pharynx, as well as afferent neurons of the brain and brainstem. This coordinated developmental sequence, between the URT and its controlling brainstem motor nuclei is largely responsible for the neuromuscular specificity necessary for proper postnatal URT control. As such, MCD in lower brainstem motor nuclei represents a critical period in the formation of peripheral URT control.

Chapter 8

Methods For Investigating Nucleus Formation and GABAergic Innervation

8.1 Motor Nucleus Formation

8.1.1 Introduction

As described in the previous chapters, MCD was assessed histologically by counting neuroblasts within each nucleus. As such, it was important to define the boundaries of NA and XII to ensure that the cells counted were indeed motoneurons to muscles of the URT. Thus, counts began only at those ages at which the nucleus appeared fully distinct in all serial sections (i.e., E16 for XII and E17 for NA). However, it was not possible to assess with routine H&E whether the nucleus was, in fact, represented by all URT motoneurons present during these stages. This was particularly important in NA where neuroblasts must migrate from the fourth ventricle to the ventrolateral adult location.

In order to determine whether motoneurons were still migrating to brainstem motor nuclei during the period of MCD novel neural tracing methods were employed. These methods employed the fluorescent carbocyanine dye, fast-DiI, to identify the location of motoneurons whose axons projected into the nerves of the URT prior to, and during, the period of MCD. Labeling of migrating motoneurons was considered possible based upon three observations: 1) motor axons in the mature NA initially project dorsomedially before "looping" ventrolaterally to leave the brainstem thus implying migration after axogenesis (Kalia and Mesulum, 1980; Grélot et.al., 1989); 2) histological studies of developing neuroblasts, including those in NA, observed axonal projections leaving the central nervous system prior to and during the period of migration (Levi-Montalcini, 1964; Heaton et.al., 1978; Brown, 1990); and 3) neural tracing studies have previously been shown to label apparently migrating neurons (Heaton and Moody, 1980; Lumsden and Keynes, 1989). These findings suggested that application of tracer to URT nerves, just distal to their exit from the brainstem, would label motoneurons still migrating to the ventrolateral NA or dorsomedial XII around the period of MCD.

8.1.2 Neural Tract Tracing

One of the most characteristic features of neurons is their interaction with neuronal and non-neuronal cells by dendritic and axonal processes. These processes, particularly axonal projections, may transmit signals to cells in the immediate vicinity of, or several feet away from, the neuronal soma. The anatomy of such projections can provide insight into the functional interactions between neurons and their target cells. However, the large distance between the nerve cell body and the target, particularly in the neuromuscular system, prevents the use of routine histological methods to demonstrate the anatomical relationship between the two cell populations. As such, methods were devised by which axons could be traced from the target tissue centrally to their cell bodies. The following sections will review the major tract-tracing methods and introduce a recent development in such neuroanatomical studies, the carbocyanine dyes.

8.1.2.1 Retrograde Neural Tracers

One of the oldest means by which neural connections have been demonstrated is by staining the neuron from process to process. Such a technique was developed over 100 years ago by Golgi and, in turn, mastered by Ramon y Cajal for his studies of the structure of the nervous system (Golgi, 1879; Strong, 1896; Ramon y Cajal, 1966). The demonstration of entire neurons has more recently been accomplished by injecting single cells with fluorescent dyes or horseradish peroxidase (HRP) (Kater et.al., 1973; Nicholson and Kater, 1973; Muller and McMahan, 1976; Sakai, et.al., 1978; Light and Durkovic, 1976). Although these techniques are useful in elucidating local projections of neurons, long axonal tracts, such as those to peripheral tissue, must be reconstructed from serial sections (Burke, et.al., 1979; Preston, et.al, 1980). Additionally, when tracing long fiber tracts, the injected dye often fades toward the distal processes preventing accurate assessment of the synaptic site (Kitai and Bishop, 1981). Thus, neuroanatomical studies

of peripheral axonal projections are not generally studied by routine histological techniques.

In the late 1960's it was noted that axons transported material from the axon terminal toward the cell body (Lasek, 1967; Watson, 1968). This observation provided the foundation of modern neural tracing techniques as researchers found that exogenously introduced proteins could be carried from muscle to the motoneuron soma. The first retrograde tracer that produced specific and reproducible results was horseradish peroxidase (Kristensson and Olsson, 1971; LaVail and LaVail, 1972). HRP is a 40kD heme-protein that catalyzes the oxidation of chromogens to a brown reaction product in the presence of hydrogen peroxide (Straus, 1974; Rennke and Venkatachalam, 1979; Warr et.al., 1981). Since the incorporation of HRP into neural tracing studies, many fluorescent substances have also been shown to be retrogradely transported by neurons (Kuypers et.al., 1977; McPheeters and Okun, 1980; Skirboll et.al., 1984; Schmued and Fallon, 1986). This has allowed the simultaneous tracing of several separate neural tracts in the same organism (Kitamura et.al., 1991; Nunez-Abades et.al., 1992).

The methodologies employed in tracing studies using HRP and similar substances largely reflect the need for a functioning retrograde transport system (LaVail and LaVail, 1974; Skirboll et.al., 1984). As such, these studies require surgery for the introduction of the tracer into the target tissue. Subsequently, the animal must survive the surgery for a long enough time to allow transport of the tracer back to the neuronal soma but short enough to prevent its removal by lysosomal degradation (Kristensson and Olsson, 1974; Dennis and Kerr, 1976). This "optimal survival time" varies based upon many factors including age of the animal, length of the pathway, types of neurons, diameter of the axons, and activity of the nervous system (see review, Warr et.al., 1981). Additionally, the anesthetic used during and after surgery can influence retrograde transport (Mesulam and Mufson, 1980). For example, the rate of HRP transport in the vagus nerve has been shown to be influenced by type of anesthetic (Rogers et.al., 1980).

The requirement for survival surgery and the factors effecting retrograde transport are particularly limiting in studies of prenatal mammalian neuromuscular development. For example, *in utero* survival surgery must be performed on the embryo or fetus for the introduction of the tracer. The tracer must be injected specifically into the muscle or muscles under study to prevent labeling of collateral pathways (Warr et.al., 1981). This is particularly difficult in small fetal muscles such as those found in the developing larynx and pharynx. Furthermore, depending on the stage studied, the muscles may be immature, represented by only primary myoblasts or lacking neuromuscular synapses (Rubinstein and Kelly, 1981). Additionally, developmental changes may significantly alter the nature of the system under study between the injection of tracer and the sacrifice of the animal. For example, if URT muscles in the rat were injected at E16 and the animal sacrificed at E19, retrograde transport would occur during the period of MCD. Thus, a large portion of the motoneurons taking up tracer at E16 may have degenerated by E19 providing erroneous data as to the nature of the motor pool at the time of injection. For these reasons, maturational changes in prenatal motoneuron populations have been difficult to study and have required the development of novel neural tracing techniques.

One means of studying prenatal motoneurons is in cell cultures of dissociated embryonic or fetal spinal cords (O'Brien and Fishbach, 1986). Such cell cultures, however, also contain sensory and inter-neurons, as well as glial cells. Thus, retrograde tracing techniques have been used to label motoneurons prior to dissociation so that they could be isolated in cell cultures (Calof and Reichardt, 1984; Kuffler, 1990). However, HRP can only be visualized in fixed and, as such, dead cells, and retrograde fluorescent tracers fade after several days (Warr et.al., 1981; O'Brien and Fishbach, 1986). This has limited the ability to study biological mechanisms in cultured embryonic neurons. Thus, led largely by Honig and Hume, researchers sought to find non-neurotoxic substances

that would retrogradely label neurons and persist in culture for several weeks (see review, Honig and Hume, 1989).

Honig and Hume (1986), recognizing the limitations of conventional neural tracers, began to explore the ability of other substances to label spinal cord neurons. Among the first substances they tried were two related carbocyanine dyes that had previously been used to study diffusion rates in lipid membranes (Sims et.al., 1974; Schlessinger et.al., 1977). Their results were "so striking that [they] did not extend [their] search much further" (Honig and Hume, 1989:p333). These dyes were shown to label neurons retrogradely by diffusion in the cytoplasmic membrane and to persist in the dissociated cultured cells for over two weeks. Additionally, Honig and Hume demonstrated that the dyes did not interfere with normal cellular function and did not cross from labeled to unlabeled membranes. Of the two dyes studied, one was found to produce superior traces: 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine, perchlorate, abbreviated DiI-C₁₈-(3), or simply, DiI.

8.1.2.2 DiI and Fast-DiI

After the finding that the carbocyanine dye, DiI, could label cells by retrograde diffusion, studies focused on determining the best methods for applying and visualizing the tracer. In Honig and Hume's (1986) original paper they injected an ethanol-based solution of DiI into the sympathetic ganglia of isolated, but biologically active, chick embryo spinal cords. The dye was then allowed to diffuse for 12-16 hours to maximize labeling. Other studies have also injected a solution of DiI or, alternatively, have placed pure crystals of the tracer on the innervated organ (Thanos and Bonhoeffer, 1987; Serbedzija et.al., 1989; Serbedzija et.al., 1990). All these studies, however, used biologically active preparations reflecting the influence of traditional HRP tract tracing protocols derived in the 1970's (LaVail and LaVail, 1975). Recall that HRP tracing requires living tissue because the protein must be actively transported to the neuronal

soma. However, DiI, although able to be internalized and actively transported *in vivo*, principally labels cells by passive diffusion in the cytoplasmic membrane (Sims et.al., 1974; Honig and Hume, 1989). Thus, in 1987, Godement and co-workers studied the ability of DiI to label cells *in vitro*, in postmortem fixed tissue (Godement et.al., 1987).

The study by Godement and co-workers (1987) showed that DiI does indeed diffuse in the membrane of aldehyde fixed tissue. They demonstrated compatibility of the dye with standard 4% phosphate-buffered paraformaldehyde but obscurement of the signal by the addition of naturally fluorescing gluteraldehyde. Additionally, rather than inject solutions of DiI, crystals of the tracer were applied directly to the cut end of the nerve or inserted in the innervated tissue. In both cases, DiI successfully traced the course of the innervated axons. A drawback to using fixed tissue, however, was the length of time needed for diffusion. Whereas living tissue could be traced in one day, in fixed tissue the dye required several weeks to several months to label the neuronal soma (see also Elberger and Honig, 1990). Furthermore, it appeared that over time, some dye could be transferred to adjacent cell membranes causing non-specific, although weaker, staining.

The ability to use fixed tissue to trace neuronal pathways avoids the constraints of survival surgery and allows for more extensive dissection and thus greater access. However, because DiI is not internalized, but rather inserts in the outer membrane, it is susceptible to artifactual spreading during routine cryostat sectioning (Honig and Hume, 1986). As such, it has been found that vibratome sections can be used for cutting traced tissue (Godement et.al., 1987; von Bartheld et.al., 1990). Additionally, spreading of the dye has been shown to occur after coverslipping and sections have been left uncoverslipped (von Bartheld et.al., 1990; Friedland et.al., 1993). Further studies on the use of DiI have shown that it can be used with fluorescent immunocytochemistry, is compatible with decalcification methods, and can be photoconverted for electron microscopy (Elberger and Honig, 1990; von Bartheld et.al., 1990).

Recently, studies have begun to shift from methodological concerns to neuro-anatomical investigations including developmental studies of cell migration and axonal projections (Thanos and Bonhoeffer, 1987; Dodd and Jessell, 1988; Serbedzija et.al., 1989; Serbedzija et.al., 1990). While no DiI studies have examined innervation of the URT, the dorsal motor nucleus of the vagus (dX) and its relationship to the vagus nerve has been investigated (Berthoud et.al., 1990).

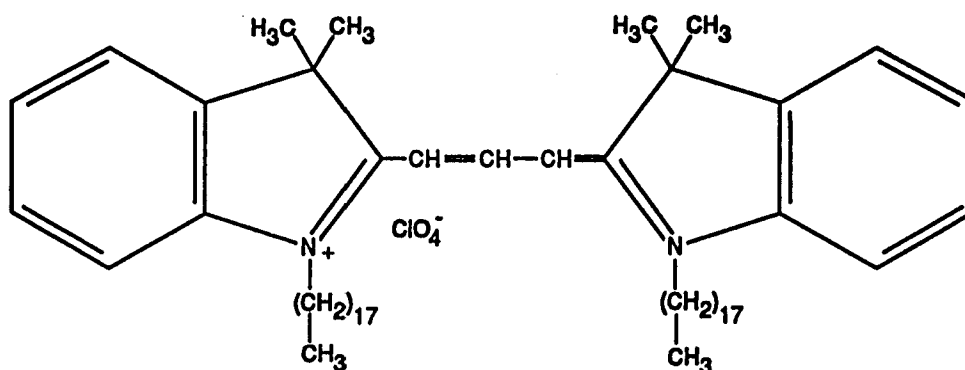
The recent increase in experiments employing DiI has prompted Molecular Probes, Inc. to modify the structure of the molecule to facilitate ease of use and reduce diffusion time. The new product has been named fast-DiI and is chemically defined as 1,1'-dilinoylel-3,3,3',3'-tetramethyl-indocarbocyanine, perchlorate. Fast-DiI incorporates a diunsaturated alkyl chain that increases its rate of diffusion in lipid membranes. This modified molecule forms a thick oil rather than the crystalline structure of the saturated DiI. The structures of DiI and fast-DiI are shown in figure 8.1. The long alkyl chains are lipophilic and responsible for insertion of the molecule into the cell membrane (Honig and Hume, 1989). Additionally, the resonance in the ring structures accounts for the fluorescence which is maximal at 565nm and 563nm for DiI and fast-DiI, respectively (Haugland, 1992). The dyes can be visualized with standard rhodamine optics or with recently developed filters specific for DiI and fast-DiI.

8.1.3 Preliminary Studies

Preliminary neural tracing studies began shortly after the protocol for studying MCD was worked out. As such, many aspects of tissue processing, such as microdissection techniques and animal handling, were well established. However, there were two methodologies of neural tracing that needed to be addressed: 1) the introduction and use of a compatible fixative; and 2) the application and subsequent visualization of DiI.

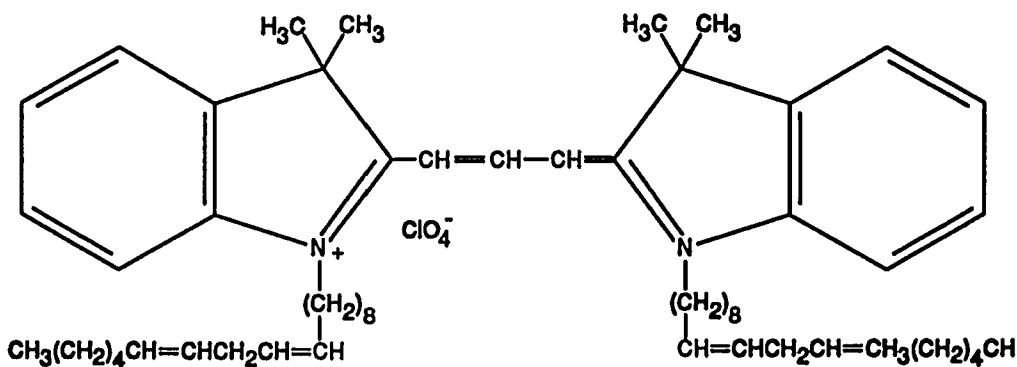
Figure 8.1
Structure of DiI and Fast DiI

DiI



1,1' dioctadecyl 3,3,3',3' tetramethylindocarbocyanine perchlorate

FAST DiI



1,1' dilinoleyl 3,3,3',3' tetramethylindocarbocyanine perchlorate

8.1.2.1 Fixation Methods

As described above, the carbocyanine dyes are useful in neural tracing because they can be applied to fixed postmortem tissue. This is particularly useful for mammalian developmental studies because it avoids the complications of *in utero* surgery.

Additionally, careful dissection of the structures under study can be performed allowing greater specificity in tracer application thus reducing extraneous label. It is important, however, that the fixative be compatible with the neural tracer and that the methods of fixation not impede the accurate analysis of the developmental phenomena under study. Such concerns lead to the use of Carnoy's fixative in MCD studies (see chapter 5) and helped define specific fixation parameters for the investigation of nucleus formation.

Pilot Studies

DiI and fast DiI are insoluble in water and this hydrophobicity helps drive the insertion of the long alkyl chains into the lipid bilayer (Honig and Hume, 1989). Additionally, ionic interactions determine the solubility of these dyes and their uniformity of diffusion within the membrane (Haugland, 1992). As such, Carnoy's fixative, which was used in the MCD study, could not be used in the study of nucleus formation because 1) Carnoy's is a dehydrating agent which removes water thus preventing the hydrophobic interactions promoting DiI insertion into the cell membrane and 2) Carnoy's is highly acidic which alters the ionic interactions that promote DiI insertion and diffusion. Thus, a relatively neutral, water-based fixative needed to be employed. A study by Godement and co-workers (1987) showed that carbocyanine dyes could be used in tissue fixed with 4% phosphate-buffered paraformaldehyde.

As discussed previously, 4% paraformaldehyde was the first fixative used for fetal brainstem fixation but did not provide adequate results for the study of MCD (see chapter 5). This was due to the inability to introduce the fixative so that initial breakdown of

subcellular components, specifically the nucleus and nucleolus, was prevented. However, for the study of brainstem nucleus formation, limited subcellular degeneration would not pose a problem. That is, analysis of neural traces does not rely on the identification of specific subcellular components but rather on gross brainstem regions comprised of populations of cells. Thus, cells could undergo some initial subcellular degeneration but still be fixed prior to the dissolution of the outer cell membrane. This would maintain a continuous lipid layer for the diffusion of DiI allowing the visualization of cell bodies and collectively, the brainstem motor nucleus. As such, it was decided to try 4% paraformaldehyde in 0.1M PBS, pH=7.4 as an initial fixative for use in DiI neural traces.

There are two methods by which fixative may be introduced to prenatal rats: 1) they could be removed from the uterus and immersion fixed; or 2) they could be vascularly perfused *in utero*. Prior experience (see chapter 5) indicated that immersion fixation produced poor results, particularly in the larger, late gestational organisms. This was retried, however, because of the less stringent fixation requirements in this study. E17 rats were removed from their mother and immersion fixed in neutral formalin. The brainstems were dissected out and cut on the cryostat at 60µm. These sections had a tattered and poorly fixed appearance and it was decided to try direct perfusion.

In utero perfusion was previously rejected as an option for the study of MCD because of possible motoneuron necrosis due to hypoxia and hypovolemia during the surgery. However, for neural tracing, as long as the cell membrane remains intact the tissue could provide viable data. Thus, E15 rats were perfused with approximately 10ml of 4% paraformaldehyde through the umbilical vessels. Specifically, the mother was anesthetized with ketamine/rompun injected intramuscularly rather than intraperitoneally to avoid the uteri. A lateral abdominal incision was made through which the uterine horn was extruded onto sterile gauze. Individual uterine sacs were opened, a 30g needle inserted into the umbilical vein, and the rat perfused with ice-cold fix. During the

perfusion the umbilical artery was cut to provide an outflow. After perfusion, the cranium was opened to expose the brainstem to subsequent immersion fixation until the specimen was used for tracing (several days to 6 months). This method provided adequate fixation and was used for all prenatal specimens from ages E13 to E17.

8.1.2.2 Neural Tracing with DiI / fast-DiI

The investigation of nucleus formation was started prior to the advent of fast DiI, and the initial experiments employed the crystalline DiI. These initial studies focused on the two most important aspects of DiI neural tracing: 1) how to apply the DiI to the nerves; and 2) how to section the tissue for fluorescence microscopy.

Pilot Studies

The advantage of using fixed tissue for neural tracing is the ability to dissect out the intended nerves without the constraints of live surgery. Thus, using the microdissection techniques described in chapter 5, the brain and brainstem of E17 rats were removed. However, certain methods needed to be adapted for formalin fixed material as it is different from tissue fixed with Carnoy's. For example, formalin fixed tissue is somewhat translucent and structures could be better distinguished by illumination from underneath the specimen. Additionally, the nerves could not be directly dissected out in the softer formalin tissue, however, by "pinching" the surrounding chondrocranium with forceps they could be isolated. Using these methods the vagus and glossopharyngeal nerves were separated from surrounding tissue just distal to the brainstem. These nerves were clearly distinguished from the more rostral facial and vestibulocochlear nerves by their prominent sensory ganglia. In contrast, the hypoglossal nerve formed from its rootlets within the hypoglossal canal and could not be isolated as a single nerve. Thus, initial studies focused on tracing the vagus and glossopharyngeal nerves.

The first attempt at neural tracing placed a single DiI crystal directly on the cut vagus and glossopharyngeal nerves. The brainstem was kept moist with a small amount of phosphate buffer and placed in a sealed container in the dark at room temperature. After two weeks, the brainstem was frozen and sectioned on the cryostat at 60 μ m. The tissue cut poorly and although no tracing to the nucleus ambiguus was apparent, some fiber tracts were seen projecting to the solitary tract. Several studies indicate similar problems with cryostat sectioned DiI material and with application of the crystalline DiI (Honig and Hume, 1986; von Bartheld et.al., 1990). Two modifications of the protocol were proposed: 1) section material on a vibratome to prevent freezing artifact; and 2) dissolve the DiI crystals in ethanol and use a filter soaked in this solution to apply the tracer.

Preliminary tests with the vibratome indicated that isolated prenatal brainstems were too small and soft to enable sectioning. Thus, rather than apply the dye to dissected brainstems and then section the tissue, whole embryos at age E15 and E17 were sectioned at 300 μ m and 500 μ m, respectively. The vagus, glossopharyngeal, and hypoglossal nerves were isolated from these thick sections and a piece of filter paper soaked in DiI/ethanol solution placed on their cut ends. The sections were moistened with buffer and kept in the dark at room temperature for two weeks. Fluorescence of the hypoglossal traces showed a fiber tract projecting toward the fourth ventricle although it appeared diffuse due to the section thickness. Coverslipping with 80% glycerol provided better distinction but diminished the fluorescent signal. Traces of the vagus and glossopharyngeal nerves showed fiber tracts to the nucleus ambiguus but were similarly diffuse. Although fiber tracts were traced to their respective brainstem motor nuclei the signal was not clear enough to allow accurate analysis of nucleus formation. It was felt that a more distinct signal could be seen if section thickness was reduced.

As these problems were being addressed, Molecular Probes Inc. released a new, faster tracing version of DiI, fast-DiI. Thus, the next set of preliminary studies focused on application of the new neural tracer and taking thinner sections of the traced

brainstems. In order to overcome the problems of sectioning the soft prenatal brainstems, Dr. Victor Friedrich of Mount Sinai suggested embedding the tissue in 4% low melting agarose. Additionally, Dr. Robert Baker of NYU provided suggestions on the use of the new methanol solubalized version of DiI. Following this advice, 5 μ l of fast-DiI was allowed to dry on a coverslip. The resultant paste was scraped together with a number 11 scalpel blade and placed on the cut vagus and glossopharyngeal nerves of an E17 formalin fixed brainstem. The brainstem was subsequently placed in fixative at 37°C. After four days, the brainstem was embedded in agar and sectioned on the vibratome at 40 μ m. Microscopic examination showed clear axons, dendrites and cell bodies in the nucleus ambiguus. However, the signal diffused after mounting with glycerol or applying the nuclear stain, DAPI. Thus, all subsequent traces were left uncoverslipped and photographed immediately.

As mentioned above, it was difficult to isolate the entire hypoglossal nerve in formalin fixed brainstems. Thus, tracing of this nerve employed a slightly different approach. The fast DiI paste was placed on the ventral surface of the lower medulla. At this level, the only fibers emerging from the brainstem are hypoglossal rootlets. In areas where no fibers entered or left the brainstem, the dye remained on the ventral surface. Where the hypoglossal rootlets were present, DiI traced these axons to the hypoglossal nucleus. Thus, although placed non-specifically, DiI was specific in labeling peripherally projecting fibers of the hypoglossal nucleus. As with nucleus ambiguus traces, hypoglossal traces were left uncoverslipped and photographed immediately for documentation.

8.2 GABAergic Innervation

8.2.1 Introduction

The assessment of MCD relied on counting large cells exhibiting distinct nucleoli within the region of NA and XII. These morphological features were chosen because they were characteristics of immature motoneurons found in all brainstem motor nuclei. However, it could not be histologically determined whether other classes of cells, particularly interneurons, could be mistaken for developing motoneurons and thus affect the assessment of MCD. This was particularly important in XII where other researchers have reported the presence of interneurons (Cooper, 1981b; Boone and Aldes, 1984; Mugnaini and Oertel, 1985; Takasu and Hashimoto, 1988). Although studies showed that the interneuron population was rather small in the adult rat (Mugnaini and Oertel, 1985), it was not known whether developmental populations of interneurons were large enough to influence significantly the observations on MCD. Thus, a study was undertaken employing standard immunocytochemical techniques to identify interneurons in the developing NA and XII.

8.2.2 Preliminary Studies

The study of GABAergic innervation used standard immunocytochemical techniques with antibodies generated against the γ -aminobutyric acid molecule. These antibodies were commercially available through Incstar and included protocols for fixation and tissue handling. Additionally, the detection of these antibodies by avidin-biotin immunoperoxidase methods was accomplished with reagents from Vector Laboratories. As such, preliminary studies focused on the implementation of these documented protocols rather than the development of novel procedures.

8.2.2.2 Preliminary Immunohistochemical Staining

There were two major concerns regarding the use of antibodies to detect interneuron populations in the brainstem. The first was a concern of any immunocytochemical study, the ability to perfuse the tissue thoroughly with a compatible fixative. The GABA specification sheet called for 4% paraformaldehyde / 0.3% glutaraldehyde in 0.1M phosphate buffer, pH=7.2. Neural tracing studies indicated that perfusion of the prenatal rats through the umbilical vessels would produce adequate fixation and no preliminary experimentation was needed to address this concern. The second consideration was specific to investigating developing systems, specifically, whether GABA was, in fact, expressed during the stages being investigated. This question was the focus of the immunocytochemical pilot studies.

Pilot Studies

In order to test for the presence of GABA in brainstem interneurons during the period of cell death, E15 and E17 rats were perfused with fixative. Two brainstems from each age were embedded in agar and sectioned on the vibratome at 50µm. These sections were collected in multi-welled immunochemical staining trays with no more than three sections per well. Avidin-biotin immunoperoxidase methods were employed using a Vectastain ABC Kit based on the studies of Hsu and co-workers (1981a, 1981b). The immunostaining protocol provided by Vector Labs was followed, however, two incubations were extended to enhance interactions between antigens and antibodies. Specifically, incubation in primary antibody was extended to 3 days at 4°C and incubation in the secondary antibody extended to 1 hour as suggested by Dr. John Morrison of Mount Sinai. Results showed staining of cells in the brainstem at both developmental stages indicating the efficacy of using antibodies to GABA to investigate interneuron populations in NA and XII. Additionally, control experiments, one in the

absence of primary antibody and the other with primary plus an excess of free γ -aminobutyric acid, showed no staining. This demonstrated the specificity of the antibody to the interneuron neurotransmitter.

The only problem encountered during the pilot study was the loss of sections during the staining protocol. Immunostaining requires the frequent transfer of section between wells for the various washing and incubation steps. Sections are often damaged and lost during these transfers and this problem is exacerbated when using small prenatal tissue. In fact, in the pilot study only 10-20% of sections survived the three day procedure. If the transfer of tissue between wells could be avoided section loss would be reduced. However, the wells were necessary because they could stain tissue in only 100 μ l of primary antibody solution. The protocol was restricted to such small volumes of incubate because the primary antibody was only effective at very low dilutions (1:30). To remedy this problem an antibody against GABA that could be used at dilutions over 1:5000 was obtained through the same company. This antibody allowed all sections from a single specimen to be stained together in large volumes of solution (>3ml) thus avoiding the use of staining wells and reducing tissue loss.

Chapter 9

Nucleus Ambiguus

Formation and GABAergic Innervation

9.1 Nucleus Formation

9.1.1 Introduction

This study sought to define the period of motoneuron migration to NA by using novel neural tracing methods. Specifically, the fluorescent dye, fast-DiI, was applied to the vagus and glossopharyngeal nerves just distal to the brainstem. In this manner, the location of neurons projecting through these nerves was determined at several prenatal ages. The results of this study established a temporal relationship between motoneuron migration to NA and the period of MCD in the same nucleus. This relationship was used to assess the accuracy of motoneuron counts in NA.

9.1.2 Experimental Design

Prenatal rats at gestational ages E13, E14, E15 and E17 were used in this study. These ages were selected based upon histological data obtained during the investigation of MCD and technical constraints of the methods employed. For example, E15 was chosen because it represents the first histological appearance of neuroblasts in NA. Likewise, E17 was studied as this is the first age in which the entire NA is histologically distinct. Based on technical limitations, E13 was the earliest developmental stage which could be adequately perfused, dissected and traced. E14 was added after preliminary results indicated dramatic changes between E13 and E15. In order to demonstrate a developmental pattern clearly, a sample size of 4 rats per age was incorporated. Unlike studies on MCD, crown-rump lengths and weights were not taken due to the substantial swelling of the specimen during perfusion.

The general methods for fixation, dissection and DiI application were described in chapter 8. There were some methods, however, specific to the study of NA formation. For example, a characteristic feature of the vagus and glossopharyngeal nerves was the prominent sensory ganglia. After the nerves were freed from the surrounding cartilage

and connective tissue, they were sectioned just distal to these ganglia. This ensured that fibers of the cranial root of the spinal accessory nerve had joined with the vagus nerve allowing the tracing of these URT motor axons as well. Additionally, during these prenatal stages, the sensory and motor components of the vagus and glossopharyngeal nerves are quite distinct. By applying tracer to the nerves distal to the ganglia both components were ensured of being traced which enabled precise assessment of brainstem development.

An important consideration when using DiI is localizing the tracer to the studied nerves. Diffusion of the dye could confound results by tracing other nearby structures. This was particularly important with the vagus and glossopharyngeal nerves as they were long enough so as to be able to bend onto the brainstem and spread the DiI to other regions. To avoid this problem, small pieces of millipore filters were laid alongside the nerves preventing their contact with the brainstem. This isolated the DiI on the vagus and glossopharyngeal nerves and prevented non-specific staining of peripheral nerves.

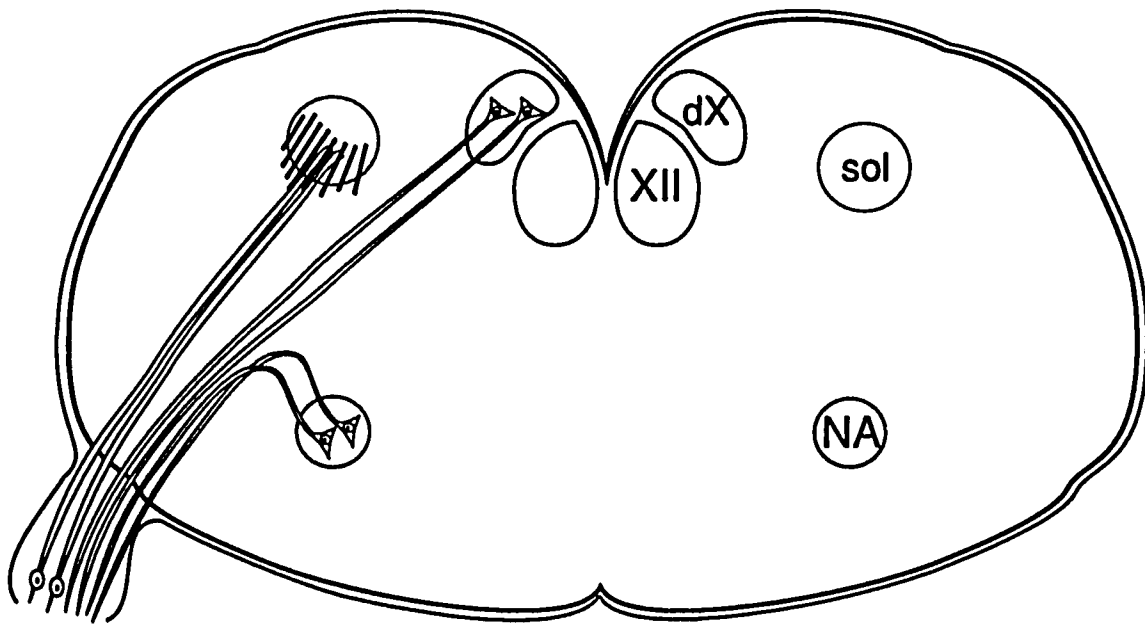
9.1.3 Results

Interpretation of the traces of the vagus and glossopharyngeal nerves requires an understanding of the central projections of these nerves. This material was reviewed in chapters 1 and 2 and a short summary is presented here. The glossopharyngeal and vagus nerves contain five specific fibers types: GSA, GVA, SVA, GVE, and SVE. The majority of afferent fibers project to the solitary tract (sol) while the efferent fibers arise from the dorsal motor nucleus of the vagus (dX) and the nucleus ambiguus. Thus, traces of these nerves should distinguish three specific brainstem regions: dorsally, sol and dX and ventrolaterally, NA (see figure 9.1). Additionally, embryologically these fiber types arise from distinct locations along the fourth ventricle. That is, sensory neurons arise from the alar plate while motoneurons arise from the basal plate. Furthermore, GVE

Figure 9.1: Illustration to demonstrate the projections of vagal and glossopharyngeal nerve fibers in the mature brainstem. Dorsolaterally are sensory projections to the solitary tract (sol). More medially are the motor projections. The principal motor fiber tract projects ventrolaterally from the dorsal motor nucleus of the vagus (dX). Axons of NA motoneurons "loop" dorsolaterally to join this tract and then extend ventrally to leave the brainstem. The following figures will refer to this diagram in order to assess and describe the results of traces of prenatal vagus and glossopharyngeal nerves.

Figure 9.1

Brainstem Projections of Traced Vagal and Glossopharyngeal Nerve Fibers



NA = nucleus ambiguus

XII = hypoglossal nucleus

sol = solitary tract and nucleus

dX = dorsal motor nucleus of vagus

motoneurons arise from the lateral-most aspect of the basal plate in contrast to the more medial genesis of SVE motoneurons.

The results of the DiI traces will be described with regard to the goals of the experiment. The first goal was to determine the locations of NA motoneurons during the period of MCD. This addressed the question of whether cells were still migrating to NA during MCD assessment and thus not included in the motoneuron counts. The second goal focused specifically on the formation of the nucleus. These results defined the stages when cells were migrating to NA and the temporal relationship of these events to histological observations of MCD.

9.1.3.1 Motoneuron Migration During MCD: E17

E17 was the first stage at which NA appeared histologically distinct and reliable cell counts could be made. As such, MCD was assessed in NA from E17 through adulthood. The vagus and glossopharyngeal nerves were traced at E17 to determine if motoneurons were still migrating to the nucleus during this period. Results are shown in figures 9.2-9.5.

Figure 9.2 shows a 40 μ m vibratome section of an E17 brainstem. Refer back to figure 9.1 for orientation and approximate neuro-anatomical locations of the brainstem nuclei. This section is from a brainstem in which the vagus and glossopharyngeal nerves were traced with fast DiI. Figure 9.3 shows the same vibratome section fluoresced under Texas Red optics to demonstrate the fast DiI. The ventral and ventrolateral borders of the brainstem are reactive for DiI because the dye diffuses within the connective tissue surrounding the tissue. However, within the brainstem, three specific regions are illuminated along with the fiber tracts of their respective neurons. In the dorsolateral region is observed the solitary tract (sol) which appears as a cluster of fibers running perpendicular to the plane of sectioning. The solitary tract fibers are not seen in a definitive cross-section because the thickness and weight of the section causes it to

literally "collapse" upon itself. Medially note the dorsal motor nucleus of the vagus (dX) which, although staining lightly, is associated with brightly fluorescent axons projecting to the periphery. In the ventrolateral brainstem is seen the nucleus ambiguus (NA) lying medial to the efferent axons of the vagus nerve. Figure 9.4 shows a higher powered view of this brainstem and neural trace. At this magnification the separation of afferent and efferent fiber tracts can be seen. Note how the NA motoneuron fibers arise from the same tract of efferent axons as those of dX. Figure 9.5 focuses specifically on NA from another specimen in which the cell bodies and their axonal and dendritic fibers are clearly demonstrated. The axons initially extend dorsolaterally before recurring to join fibers of dX to form the vagus and glossopharyngeal nerves.

9.1.3.2 Nucleus Formation: E13 through E15

The earliest stage at which motoneuron migration could be assessed was E13. This was due to the small size of the brainstem and the fragility of such tissue after vibratome sectioning. Using fast DiI, the vagus and glossopharyngeal nerves were traced at this stage and on the two subsequent gestational days. Results of these traces are shown in figures 9.6-9.16.

Figure 9.6 shows a vibratome section of an E13 brainstem. Note the dramatic difference in gross morphology between this stage and that at E17 (figure 9.2). Indicated for orientation are the classic landmarks and the exit of the vagus and glossopharyngeal nerves. Figure 9.7 shows the same section fluoresced for DiI. In these small specimens, a lot of non-specific diffusion of DiI along the border of the brainstem is seen. The most prominent, specifically traced feature at this stage is a medially directed tract extending to the midline. Examining another specimen at higher power shows this tract projecting to the medial-most border of the basal plate (figures 9.8 and 9.9). Laterally can be seen the solitary tract and an indistinct clustering in the ventrolateral brainstem representing the early stages of NA. Closer examination of this trace shows the medially directed

tract to contain neuronal bodies with an additional group of neuronal bodies seen descending from the lateral border of the basal plate (figure 9.10). This pattern is seen more clearly in a trace in which the neurons were lightly labeled (figure 9.11). In this section NA is seen in the lower left and two distinct groups of neurons appear to be migrating toward NA from the medial tract and the dorsolateral basal plate. These cells must be motoneurons because they arise from the basal plate and they have sent axons out of the brainstem through the vagus and glossopharyngeal nerves. The latter point is attested to by the specificity of fast DiI, that is, only cells projecting peripherally can be illuminated by peripheral application of the dye.

By E14 the brainstem began to appear more mature. Figures 9.12 and 9.13 show sections of the left side of an E14 rat brainstem. Note that the prominent medial migrating group of cells has largely disappeared. Furthermore, the nuclei associated with the vagus and glossopharyngeal nerves are more distinct. The dorsally located sol and dX are evident as is the ventrolateral NA. Figure 9.14 shows a higher magnification of the ventrolateral region in which some cells can still be seen migrating ventrally toward NA.

By E15, no more migrating cells are seen in the brainstem (figure 9.15). Distinct projections of vagal and glossopharyngeal fibers can be seen along with their respective fiber tracts. On high power, no cells are apparent outside the region of NA and the axons of NA motoneurons can be seen joining with those from dX (figure 9.16). These axons initially project dorsolaterally before recurring as was seen in E17 brainstems. There were no major differences between E15 and E17 tracing patterns and thus E16 was not examined.

Figure 9.2: Vibratome section through an E17 brainstem. Fast-DiI was placed on the vagus and glossopharyngeal nerve just after emerging from the brainstem (arrow). The mature location of NA is indicated by the number "1" (see below). For further orientation see figures 2.2 and 9.1, and chapter 4. (unstained, 50x)

Figure 9.3: The above E17 brainstem section fluoresced for visualization of fast DiI. Three distinct regions were specifically traced by the dye: NA (1); dX (2); and sol (3). The following figures provide closer views of the E17 DiI traced nucleus ambiguus. (fast-DiI, 50x)

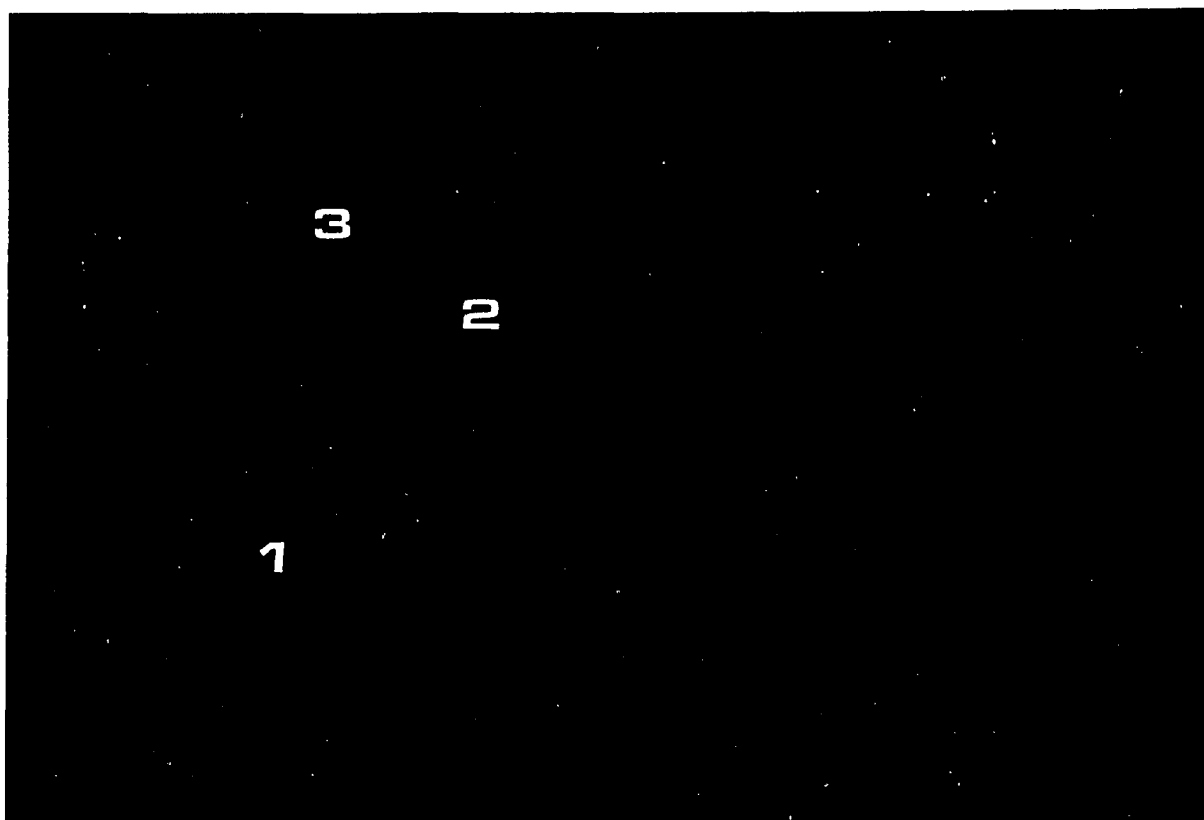
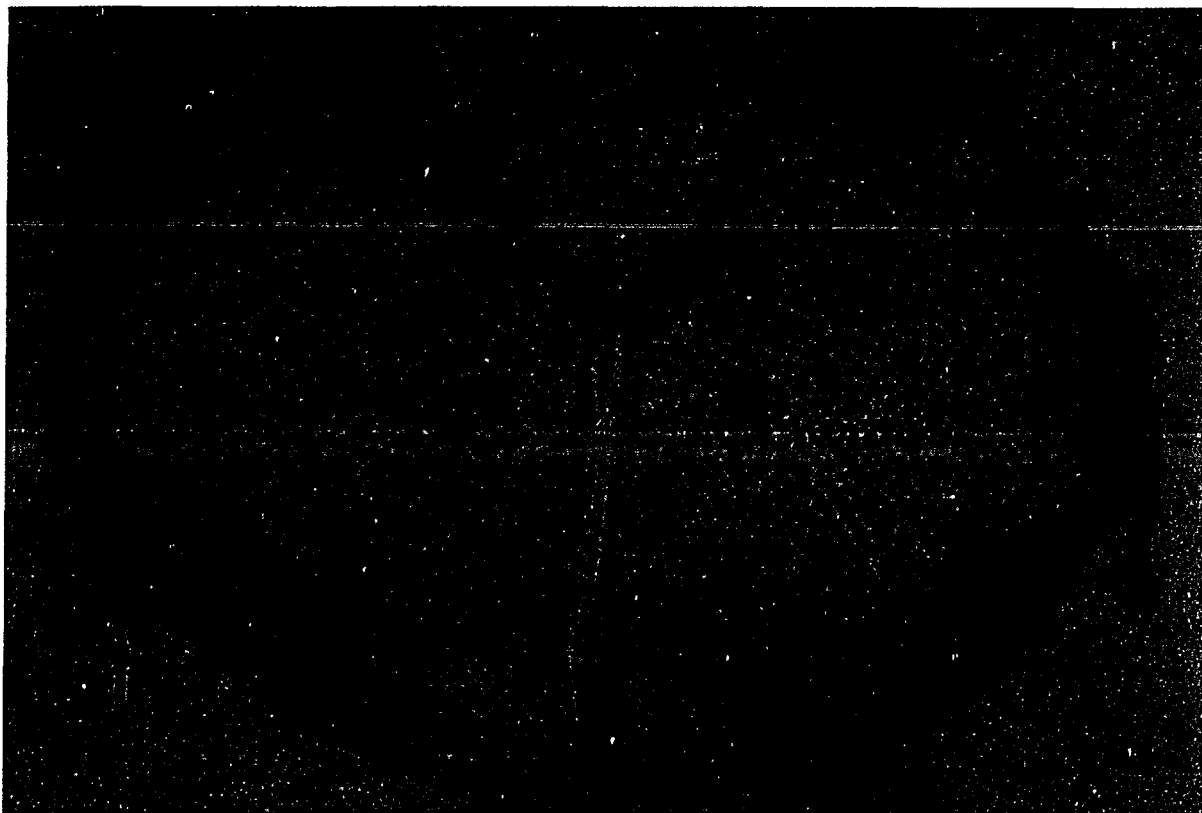


Figure 9.4: Closer view of figure 9.3, trace of the vagus and glossopharyngeal nerves.

Bottom of figure is at right edge of page (i.e., rotate 90 degrees). NA (1), dX (2) and sol (3), and the fiber tracts projecting to and from them, can be clearly seen extending to the border of the brainstem (lower left of photograph) where the nerve emerges. No cells were seen at this stage migrating to NA. (fast-DiI, 125x)

Figure 9.5: NA in another section of an E17 DiI-traced brainstem. Bottom of figure is at right edge of page (i.e., rotate 90 degrees). The nucleus appears as a cluster of DiI labeled neurons (compare to histology, figure 6.12). Axons from the motoneurons can be clearly seen projecting dorsolaterally to the main motor fiber tract (upper left of photograph) before coursing ventrolaterally to leave the brainstem in the vagus and glossopharyngeal nerves (edge of brainstem out of frame). (fast-DiI, 250x)



Figure 9.6: Transverse section through an E13 brainstem at the level of NA. Fast DiI was placed on the vagus and glossopharyngeal nerves just distal to the brainstem (arrow). The fourth ventricle is indicated for orientation (v). For further orientation see figures 2.2, 6.2, and 7.2, and chapter 4. (unstained, 80x)

Figure 9.7: The above E13 brainstem fluoresced for fast-DiI. The dominant feature of this trace is the medially directed tract (arrow) extending to the midline of the brainstem. This represents specific tracing of the nerve fibers in contrast to the non-specific diffusion in the connective tissue along the border of the brainstem (left side of photograph). (fast-DiI, 80x)

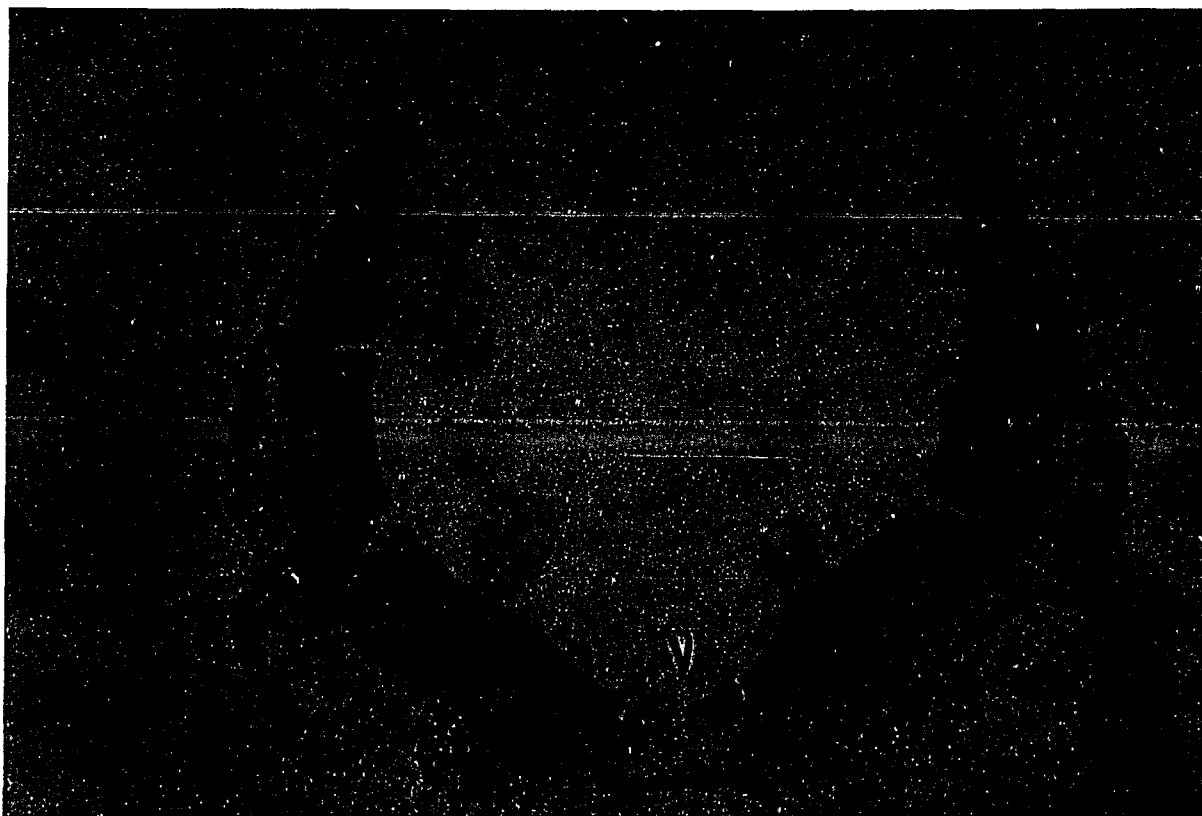


Figure 9.8: Closer view of the left side of an E13 brainstem. This figure is for interpreting the trace below and indicates the fourth ventricle (v) and the vagus and glossopharyngeal nerves where the fast-DiI was placed. The midline is just below the "v" indicating the ventricle. (unstained, 125x)

Figure 9.9: The above E13 brainstem section fluoresced for fast-DiI. The developing NA (1) and solitary tract (3) are indicated. The large arrow points to the medially directed tract while the small arrow indicates a lateral group of cells which may contribute to dX (labeled "2" in figures 9.3, 9.4) or NA. (fast-DiI, 125x)

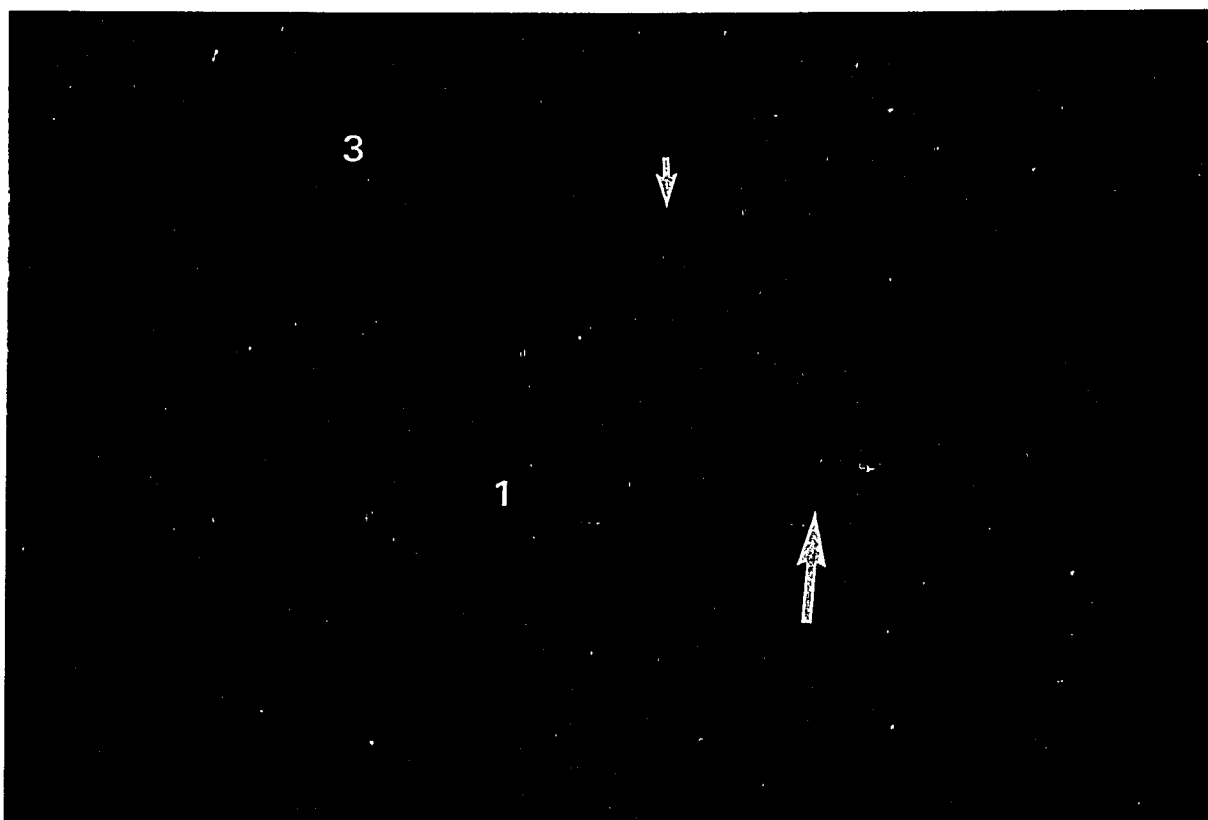


Figure 9.10: Closer view of the medial tract in an E13 vagus and glossopharyngeal DiI trace. As in the previous figures, the dye was placed on the nerve at the lower left corner of the figure and the midline of the brainstem is at the right edge. The region of NA (1) is partly obscured by the heavily labeled tract. The tract can be seen to be composed of neurons as cell bodies are seen along the periphery (large arrow). The lateral group of neurons (small arrow) can be seen apparently migrating ventrally from the ventricle to NA. (fast-DiI, 250x)

Figure 9.11: The same view of an E13 DiI trace as the above figure. In this trace the medial tract was not as densely labeled. The developing NA (1) is more apparent and the medial tract (large arrow) can be clearly seen to be comprised of cells extending their axons toward the main fiber tracts (left side of figure). The lateral group of cells (small arrow) is also apparent migrating ventrally toward NA. (fast-DiI, 250x)

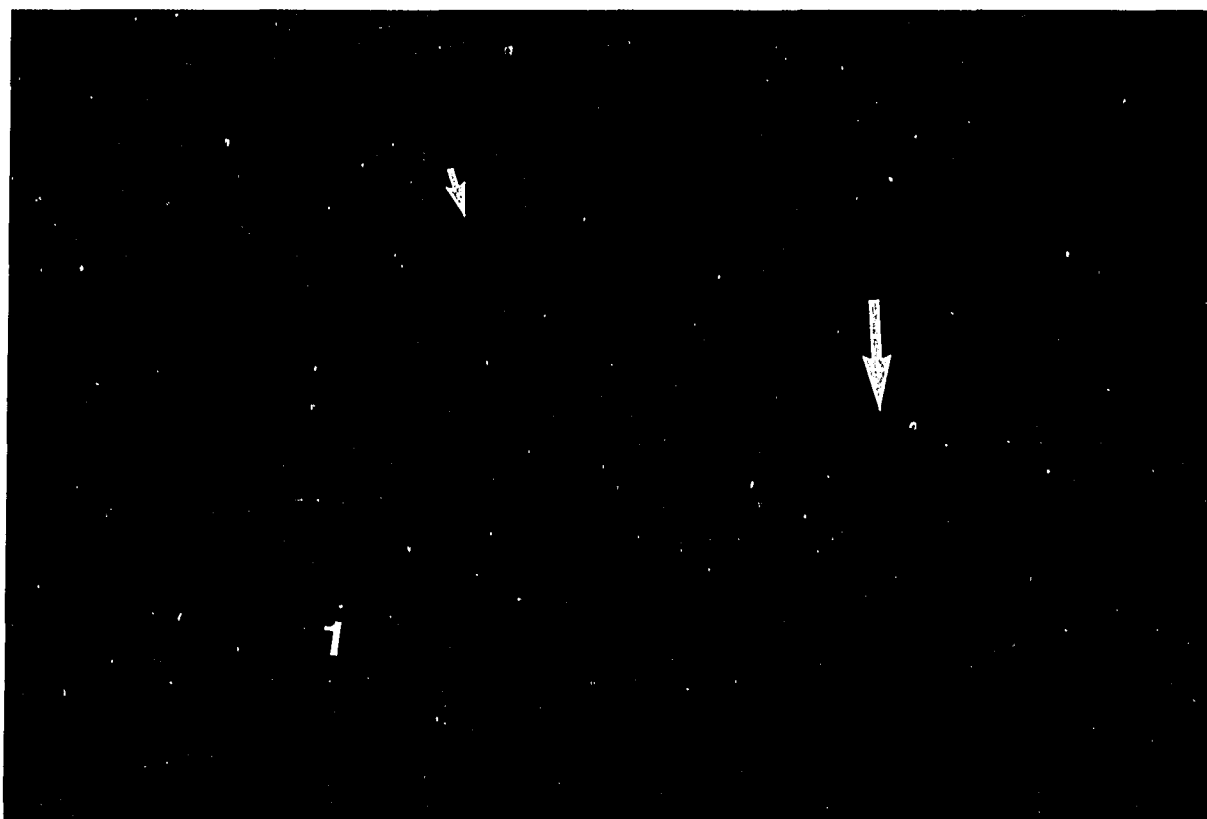
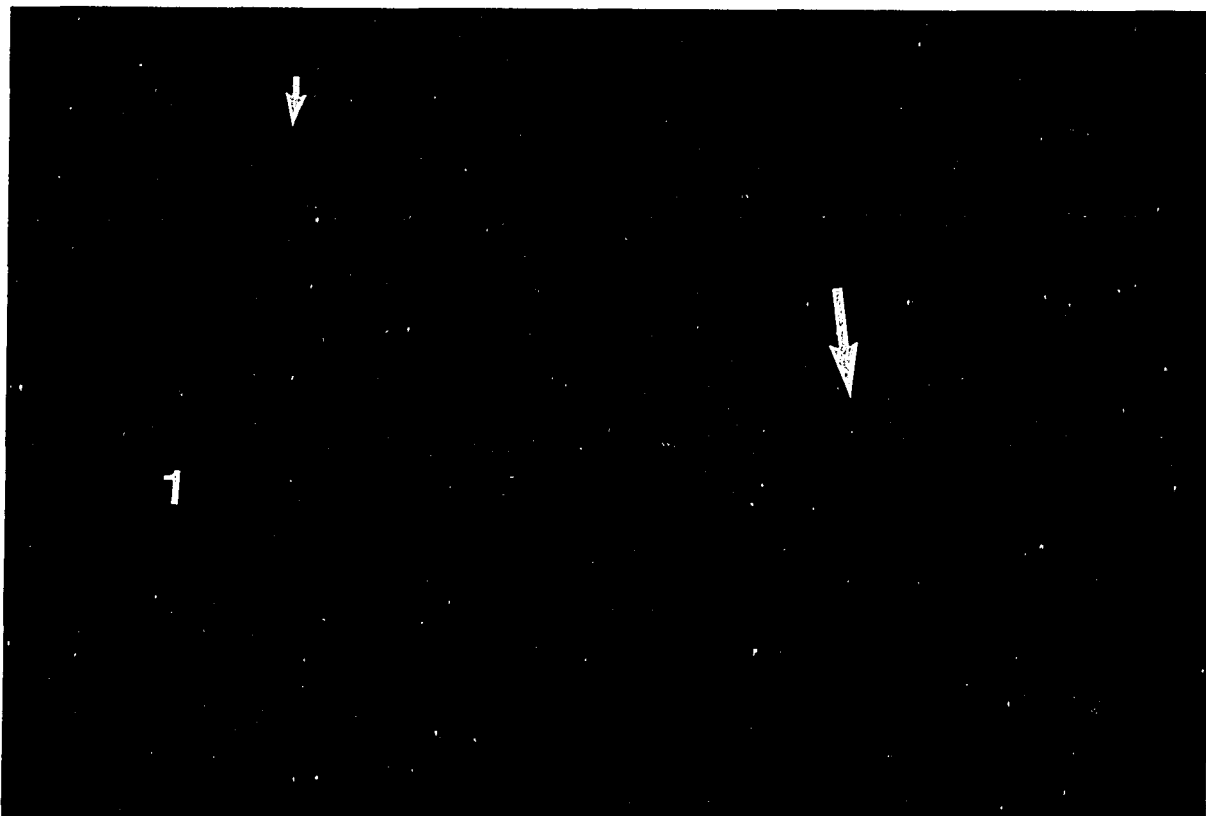


Figure 9.12: Vibratome section of an E14 brainstem at the level of NA. This figure is for orientation of the below fluoresced trace. Indicated is the fourth ventricle (v) and the mature location of NA (1). The midline of the brainstem is on the right side of the photograph extending obliquely from the "v" label to the edge of the figure. (unstained, 125x)

Figure 9.13: The above E14 brainstem section fluoresced for fast-DiI. The three regions characteristic of the distribution of the mature vagus and glossopharyngeal nerves are beginning to be defined (refer to figure 9.1). Indicated is NA (1), dX (2) and sol (3). Some cells can still be seen between dX and NA indicating the continued migration of neurons and formation of NA. (fast-DiI, 125x)

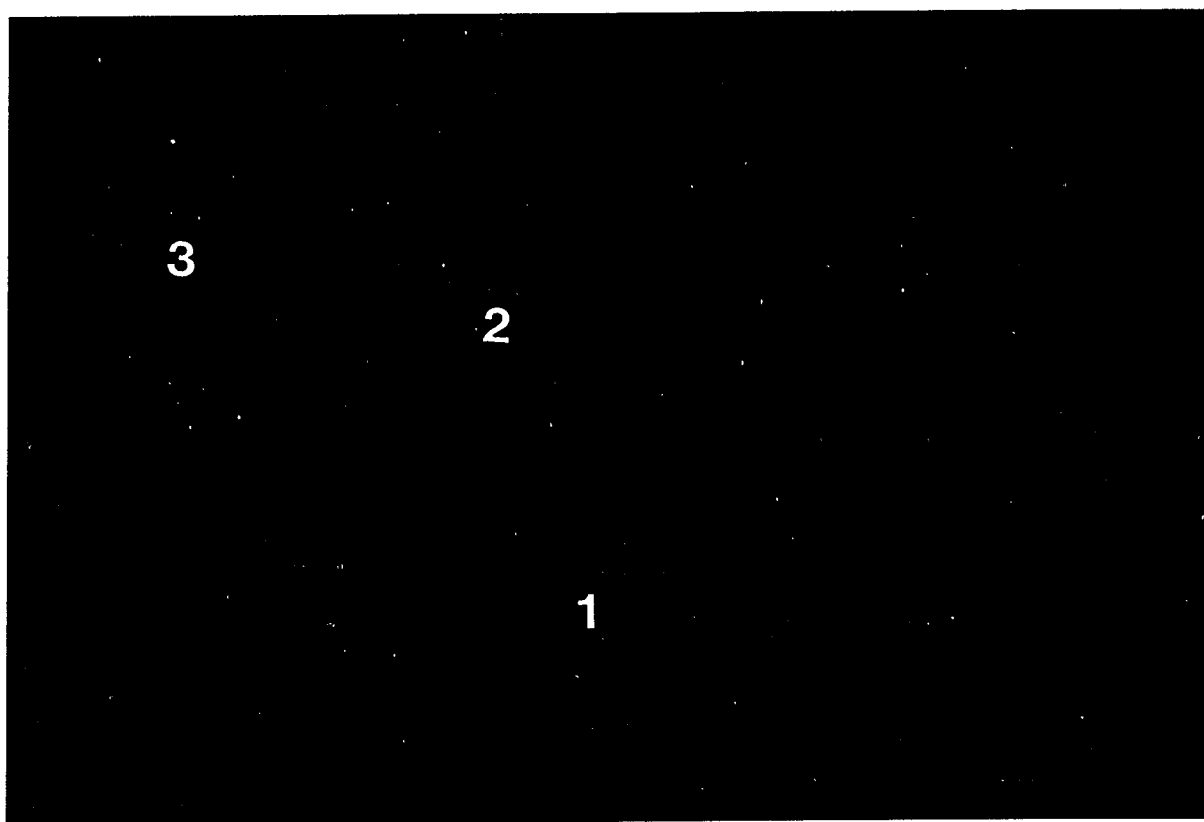
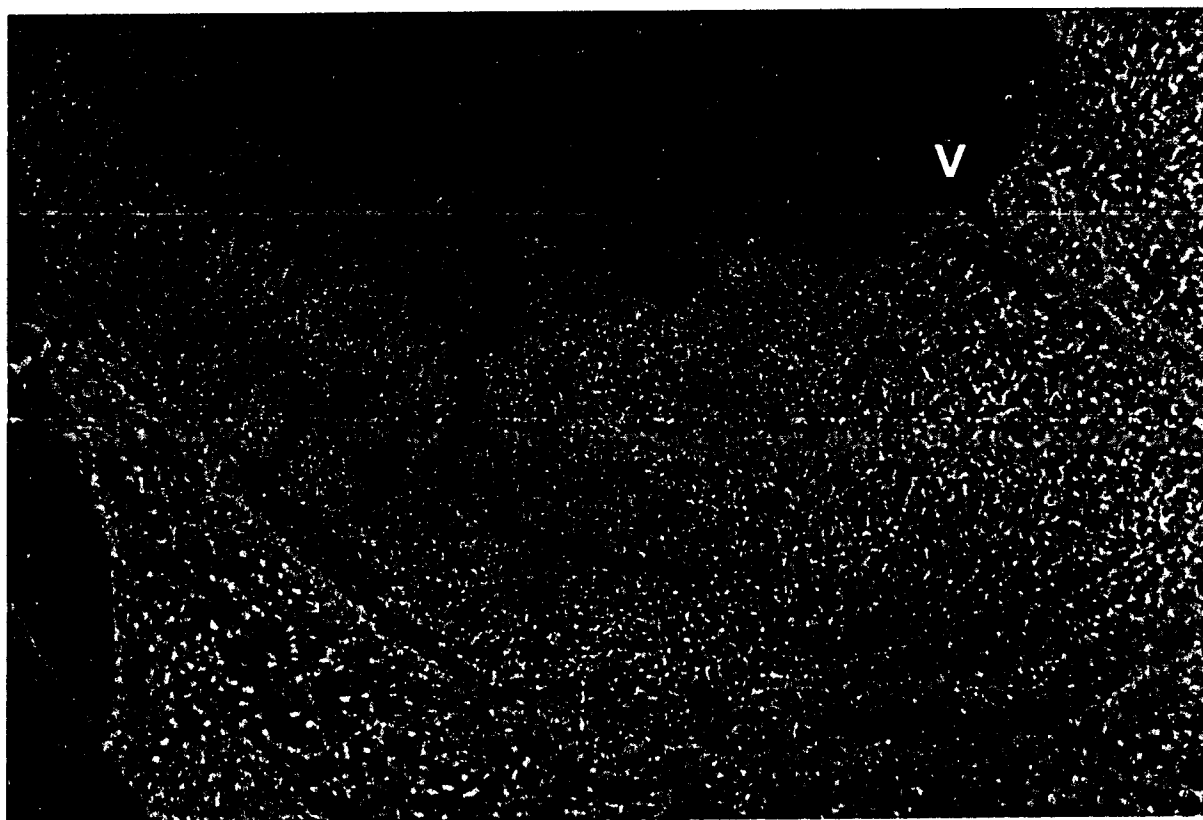


Figure 9.14: A closer view of the region of NA (1) and dX (2) in an E14 traced brainstem. Some cells can still be seen below dX migrating toward NA (arrow). This trace indicates that some ambigual motoneurons are in their mature location by E14 although they were not seen by routine histology (see figure 6.5). (fast-DiI, 250x)

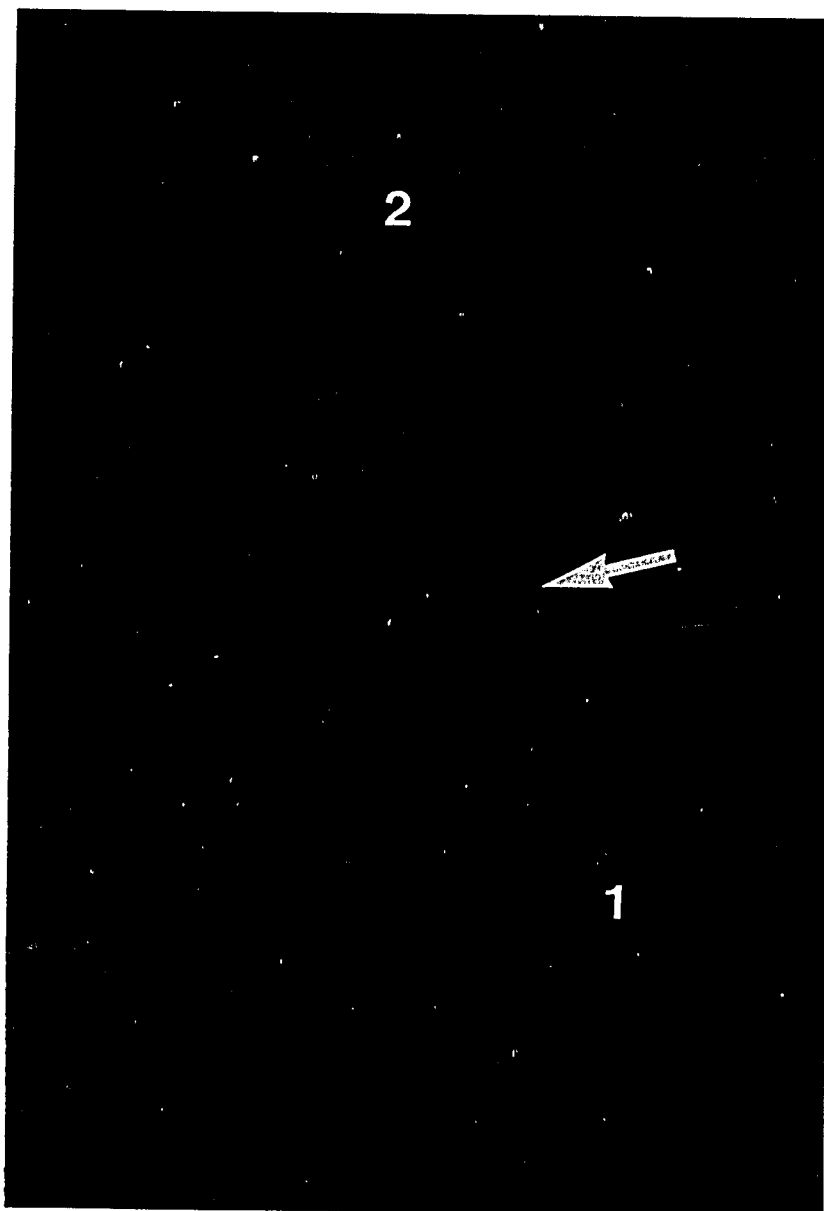
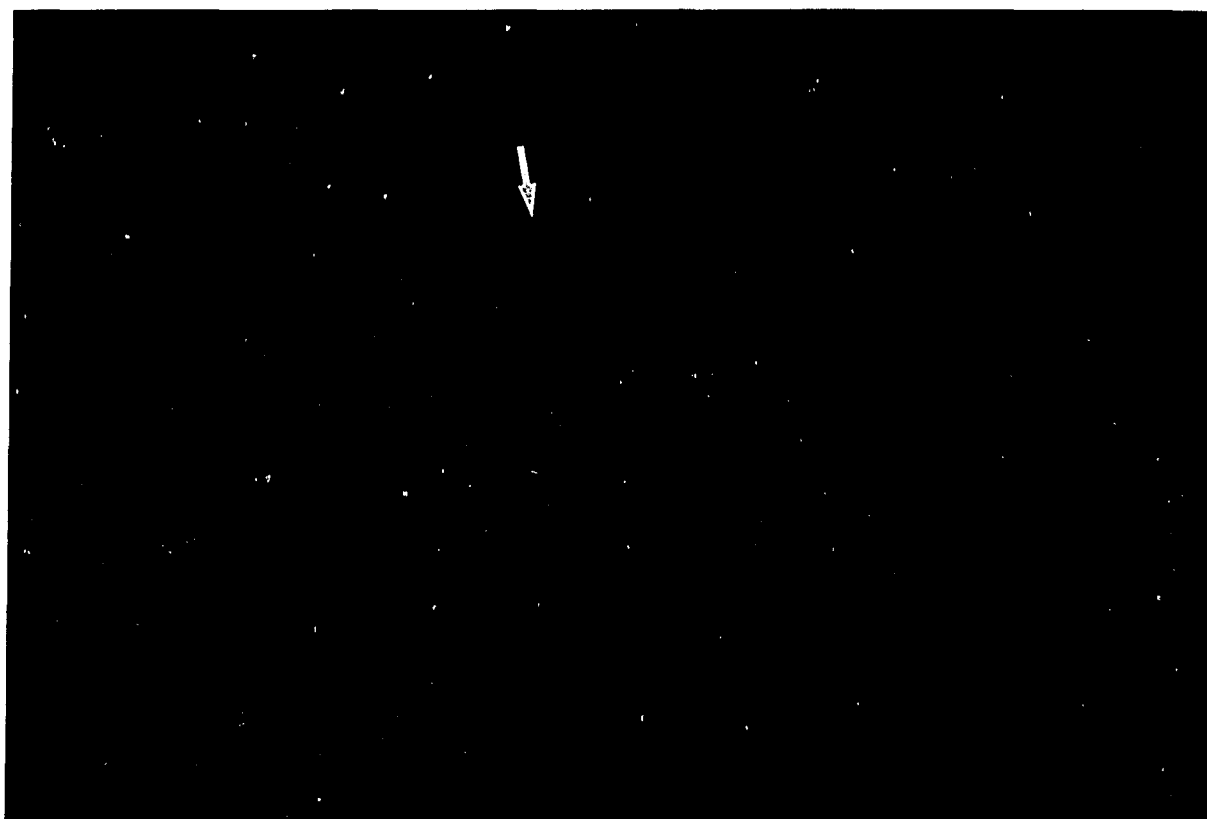
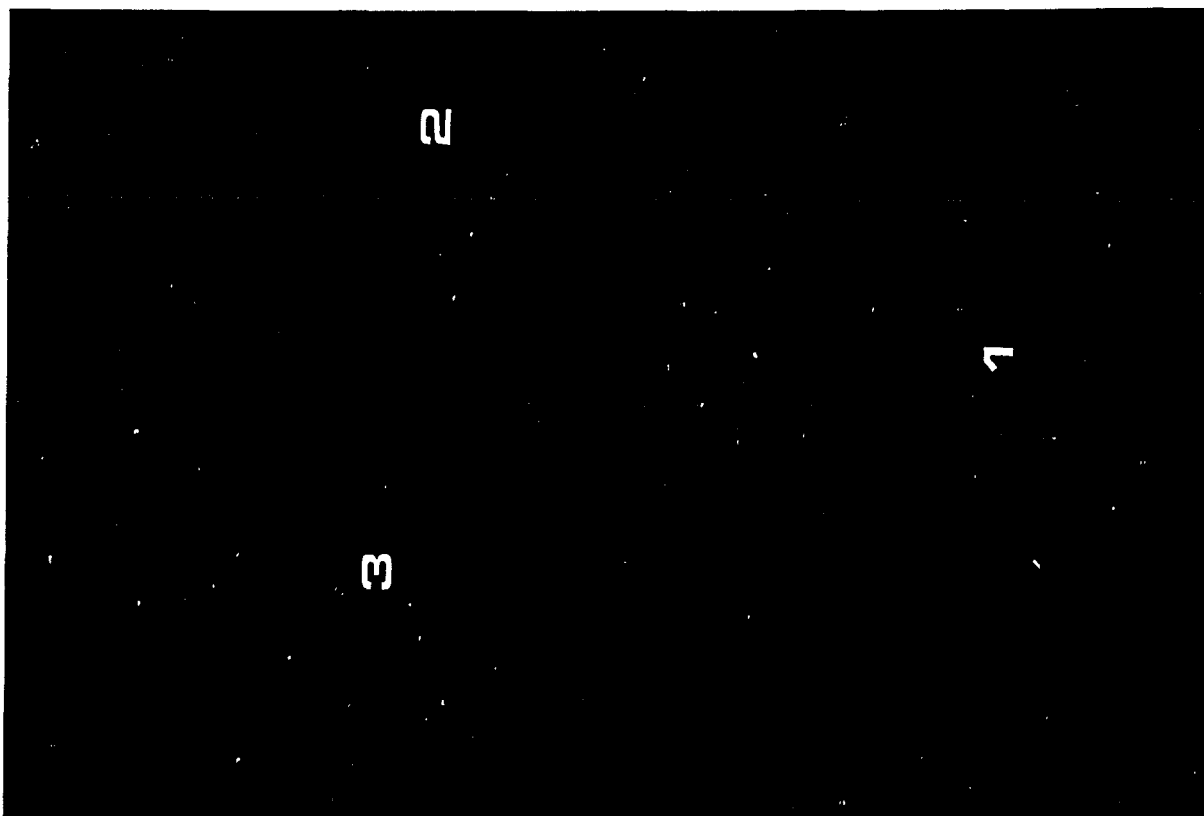


Figure 9.15: The left side of an E15 brainstem with the vagus and glossopharyngeal nerves traced with fast-DiI. Bottom of figure is at right edge of page (i.e., rotate 90 degrees). The three regions of distribution of the mature nerves can be seen : NA (1), dX (2), sol (3). Refer to figure 9.1 for further orientation. No cells are seen outside NA indicating that migration of ambigular motoneurons is complete by this stage. (fast-DiI, 125x)

Figure 9.16: NA in an E15 DiI-traced brainstem. Bottom of figure is at right edge of page (i.e., rotate 90 degrees). Although the cells are not as clear as in the E17 NA (figure 9.5) they clearly form a well-defined NA. Motor fiber tracts are seen above NA and some connections between NA motoneurons and these tracts can be seen (arrow). No cells are seen outside the NA indicating that migration is complete and the nucleus is fully formed. This is the first stage at which NA was seen histologically although it had a diffuse appearance (see figures 6.8 and 6.10). (fast-DiI, 250x)



9.1.4 Discussion

DiI tracing of the vagus and glossopharyngeal nerves were carried out in the prenatal rat to investigate aspects of NA development. The use of DiI allowed fixed postmortem tissue to be studied which enabled isolation and tracing of the ambigular nerves and the investigation of nucleus formation at specific developmental stages. The ages studies were chosen so as to address two issues: 1) is motoneuron migration to NA finished by the onset of MCD; and 2) when is NA fully formed and can migration patterns be determined.

9.1.4.1 Motoneuron Migration During MCD: E17

The results of these traces, as demonstrated in figures 9.2-9.5, show that the organization of the brainstem into discrete neuronal populations appears complete by E17. This finding conforms with the observations of NA organization as seen with routine histological methods (see chapter 6). The regions elucidated with DiI were NA, sol, and dX and were found in mature locations conforming with present understanding of rat brainstem neuroanatomy (Paxinos, 1985). No cells were observed migrating to NA, or other vagal and glossopharyngeal nuclei, at this stage. As such, at the onset of assessment of MCD in NA, no cells are located outside the motor nucleus. Thus, motoneuron counts at the early stages of MCD included all motoneurons associated with NA.

9.1.4.2 Nucleus Formation: E13 through E15

As NA appeared fully formed by E17, DiI was used at earlier stages to determine if the migration of neurons to NA could be seen. It had previously been established in several studies that neurons extend axonal processes to the peripheral nerve during migration and thus such cells could be seen by peripheral nerve tracing (Levi-Montalcini, 1964; Heaton et.al., 1978; Heaton and Moody, 1980; Lumsden and Keynes, 1989;

Brown, 1990). The earliest age studied was E13 as it represented the smallest specimens in which adequate perfusion, dissection, and sectioning could be performed. In fact, sections of E13 and E14 brainstems were extremely fragile and often cracked after mounting on the slide (see figures 9.6 and 9.12). These early traces were also less distinct than those at later stages as the section thickness was relatively greater and fluorescing structures often exhibited considerable overlap (compare figures 9.4 and 9.14). However, gross patterns of nucleus formation could be discerned and migration of NA motoneurons was observed. The observed formation of NA can be discussed with respect to two separate developmental concerns: 1) the location along the basal plate from which NA motoneurons arise; and 2) the timing of migration and formation of NA relative to neurogenesis.

Motoneurons in the brainstem arise along the ventromedial neural tube at the basal plate. As indicated in chapter 2, this plate gives rise to three columns of cells containing GSE, SVE, and GVE neuroblasts. These columns have traditionally been considered to be distinct, with the SVE (i.e., NA) neuroblasts arising between the other two columns (Smith and Williams, 1984; Sadler, 1985; Williams et.al., 1989). However, several studies have indicated that SVE motoneurons may arise along with, or even medial to, GSE motoneurons (Kimmel, 1940; Windle, 1970; Brown, 1990). The present study supports the latter observation that SVE motoneurons arise from the most medial portions of the basal plate. This is particularly evident in figure 9.9 in which the DiI positive neurons are found at the dorsal midline of the brainstem. In addition, considerable overlap between NA neuroblasts and the GSE cell column was seen in specimens in which the hypoglossal nerve was inadvertently traced by diffusion of the dye from the vagus nerve (not shown). As the NA neuroblasts migrate to the mature brainstem location they pass lateral to the GSE neurons which do not migrate. Observations at this developmental stage may account for the traditional view that SVE neurons arise as the middle cell column.

While NA is considered the motor nucleus to muscular derivatives of the third through sixth branchial arches, studies have also demonstrated a considerable contribution to innervation of the thoracic and abdominal viscera (Coil and Norgren, 1979; Kalia and Mesulam, 1980b; Takayama et.al., 1982; Stuesse, 1982; Rogers and Hermann, 1983; Housley et.al., 1987). This indicates the presence of GVE neurons in NA; neurons which arise embryologically from the lateral portion of the basal plate. The presence of parasympathetic motoneurons in NA explains the findings in E13 and E14 brainstems of a second population of migrating cells. These cells were observed descending from the ventricular layer dorsal to NA and considerably lateral to the SVE and GSE neuroblasts (figures 9.10, 9.11, and 9.14). Thus, the different populations of NA motoneurons arise from distinct locations along the basal plate.

The present study indicates that migration to NA ceases by E15 as no cells were seen outside the nucleus at this stage. This would imply that neurogenesis of NA motoneurons is likewise complete by this stage. Studies on the generation of rat NA motoneurons, while indicating that neurogenesis is indeed complete by E15, differ in their assessment of the peak birthdate of these cells. While Phelps and co-workers (1990) indicated E10-E11 as the peak generation time, Altman and Bayer (1980) claimed a peak generation of motoneurons in NA at E14 (age nomenclature corrected to conform to this study). The results of the present DiI study indicate that by E14 NA is almost fully formed and very few neuroblasts are outside the nucleus (figure 9.14). In addition, at E13, many neurons have arrived in NA as well (figure 9.11). Thus, the results of Phelps and co-workers, that NA motoneurons arise about E10-E11, are more likely to reflect the actual peak period of neurogenesis. Their study also indicated that SVE neurons arose slightly earlier, although generally during the same period, as compared to GVE neurons. This may account for the observation of earlier termination of SVE migration as compared to GVE cell migration. That is, migration of neurons from the medial basal plate (i.e., SVE neurons) was observed at E13 but not at E14, while

migration from the lateral plate (i.e., GVE neurons) was seen through E14 (figures 9.11 and 9.14).

These results indicate that after neurogenesis, NA motoneurons migrate to the mature brainstem location through E15. Migration occurs along two separate routes, one from the medial basal plate and one from the lateral basal plate. The medial group represents SVE neurons, which contrary to convention, arise most medially, along with the GSE neurons. The lateral group descends from the basal plate and probably contributes GVE neurons to both dX and NA. The medial group appears to finish migrating by E13 while the lateral group continues through E14. NA appears fully formed at E15 with little change through E17. Thus, at the time MCD was assessed, no NA motoneurons were outside the nucleus either being generated or migrating.

9.2 GABAergic Innervation

9.2.1 Introduction

The histological techniques used for the assessment of MCD were non-specific, that is, they stained all neuronal and nonneuronal cells in the brainstem. Thus, counts were made based on a collection of morphological characteristics describing immature motoneurons but excluding most other cells. The principle features used to distinguish motoneurons was their location within NA and their large size. It was unclear, however, if other neurons found in NA could have been large enough to be included in the motoneuron counts. In particular, of concern was the presence of interneurons within the compact formation of NA. In order to determine the relative population of these cells with respect to motoneurons in NA, brainstems were stained specifically for interneurons. This was accomplished by immunocytochemical methods using an antibody directed at the interneuron neurotransmitter, γ -amino butyric acid (GABA). Results from this study were used to determine the effects of including interneurons in motoneuron counts on the observed course of MCD in NA. Additionally, this study demonstrated the establishment of GABAergic innervation in NA during the period of naturally occurring cell death.

9.2.2 Experimental Design

The observations on the course of MCD indicated that E16 through E20 represented a period of dynamic change in URT motoneuron populations. As such, the investigation into GABAergic innervation focused on the extremes of this period and studied the NA of E16 and E20 prenatal rats. Although a sample size of $n=2$ was originally suggested, a repetition of the experiment to confirm initial findings resulted in a final sample size of $n=4$ at E16 and $n=8$ at E20. The methods followed were described in chapter 8 and were used in the initial and repeated staining procedures.

9.2.3 Results

Unlike the neural tracing study, the investigation of GABAergic innervation of the NA had only one goal. The goal was to determine the relative number of interneurons to motoneurons in NA. This would indicate the effect of counting interneurons along with motoneurons on the observed course of MCD. However, in addition to labeling interneuron cell bodies, GABA-positive fibers were also shown. This allowed the unanticipated demonstration of synaptic input to motoneurons of NA. Thus, as in the DiI study, the results will be presented in two parts: 1) the relative population of interneurons to motoneurons in NA during the period of MCD; and 2) the establishment of GABAergic innervation patterns in the developing NA between E16 and E20.

9.2.3.1 Relative Interneuron Populations

The results of this study are shown in figures 9.17-9.25. Figure 9.17 shows a control section of a prenatal rat brainstem at the level of NA. This was a control in which primary antibody was replaced in the incubate with normal serum but all other steps remained unchanged. The arrows indicate the location of NA. Note that the brainstem is uniformly unstained and exhibits a homogeneous appearance with no nuclei readily apparent. Figures 9.18 and 9.19 show the left sides of stained E16 and E20 brainstems in the presence of primary antibody. The brainstems show GABA immunoreactivity in virtually all regions, however, NA is conspicuous at both ages by its relative lack of staining.

Examination of an E16 NA under higher power more readily demonstrates the relative lack of immunoreactivity within the nucleus (figure 9.20). Small GABA-positive cells can be seen around NA but the majority of immunoreactive structures are dense fiber tracts cut in cross-section. In order to determine the relative population of motoneurons the section was fluoresced under UV light to illuminate nuclei stained with

DAPI (figure 9.21). DAPI is a fluorescent stain that illuminates the cell nucleus.

Comparing figures 9.20 and 9.21 shows that NA contains many cells with large nuclei that are not reactive for GABA. Evidence from histological and neural tracing studies indicates that these cells must be motoneurons. Thus, during the period when MCD was first assessed, i.e., around E16, the motoneuron population was far greater than the population of interneurons.

By the end of MCD, the relative populations of motoneurons and interneurons does not appear to have changed. Figure 9.22 shows NA in an E20 rat. GABA-positive cells are scarce as in the E16 NA, but there appears to be an increase in immunoreactive fibers within the nucleus. When this section was fluoresced, large motoneuron nuclei were seen to correspond to the non-reactive regions of NA (figure 9.23). Thus at E20, as at E16, motoneurons far outnumbered interneurons indicating a relatively constant numerical relationship throughout the period of MCD.

9.2.3.2 GABAergic Innervation

Though the relative number of interneurons remains constant between E16 and E20, the presence of immunoreactive fibers within NA appears to have increased. Figures 9.24 and 9.25 show GABA immunoreactivity in NA at E16 and E20, respectively. The E16 NA lacks any prominent GABA-positive fibers although many such fibers are evident outside the nucleus. In contrast, the E20 nucleus contains many immunoreactive fibers partly obscuring the extent of the nucleus, particularly along the ventromedial pole. The fibers appear to form "cells" around spaces of no immunoreactivity. As seen in figures 9.22 and 9.23, these spaces correspond to large nucleated cells. Thus, between E16 and E20 there appears to be an increase in the number of GABA-positive fibers projecting into NA surrounding the developing motoneurons.

Figure 9.17: Control section through an E20 brainstem at the level of NA. Primary antibody for GABA was omitted from the incubate and replaced with normal serum. Note that the brainstem appears uniformly light brown with no distinction of different regions apparent. (GABA control, 50x)



Figure 9.18: Left side of an E16 brainstem stained for GABA. Regions of positive stain appear throughout the brainstem (compare to control section, figure 9.17). NA is apparent by its relative lack of stain (arrow). For further orientation see figure 2.2 and chapter 4. (GABA, 100x)

Figure 9.19: Left side of an E20 brainstem stained for GABA. As at E16 (above), regions of positive stain appear throughout the brainstem. NA, although less distinct than at E16, is again apparent by its relative lack of stain (arrow). The differences in color between this and the above figure are due to processing during development and do not reflect differences in immunoreactivity. (GABA, 80x)



Figure 9.20: NA in an E16 GABA stained brainstem. The nucleus is apparent by its relative lack of stain. No interneurons are apparent within NA although some are seen at the periphery (e.g., arrow). (GABA, 500x)

Figure 9.21: The above NA fluoresced to indicate DAPI labeled nuclei. Cell nuclei appear as pale blue ovals and are densely packed in NA. These cell nuclei represent the motoneurons of NA and far out-number the relatively small interneuron population. (DAPI, 500x)

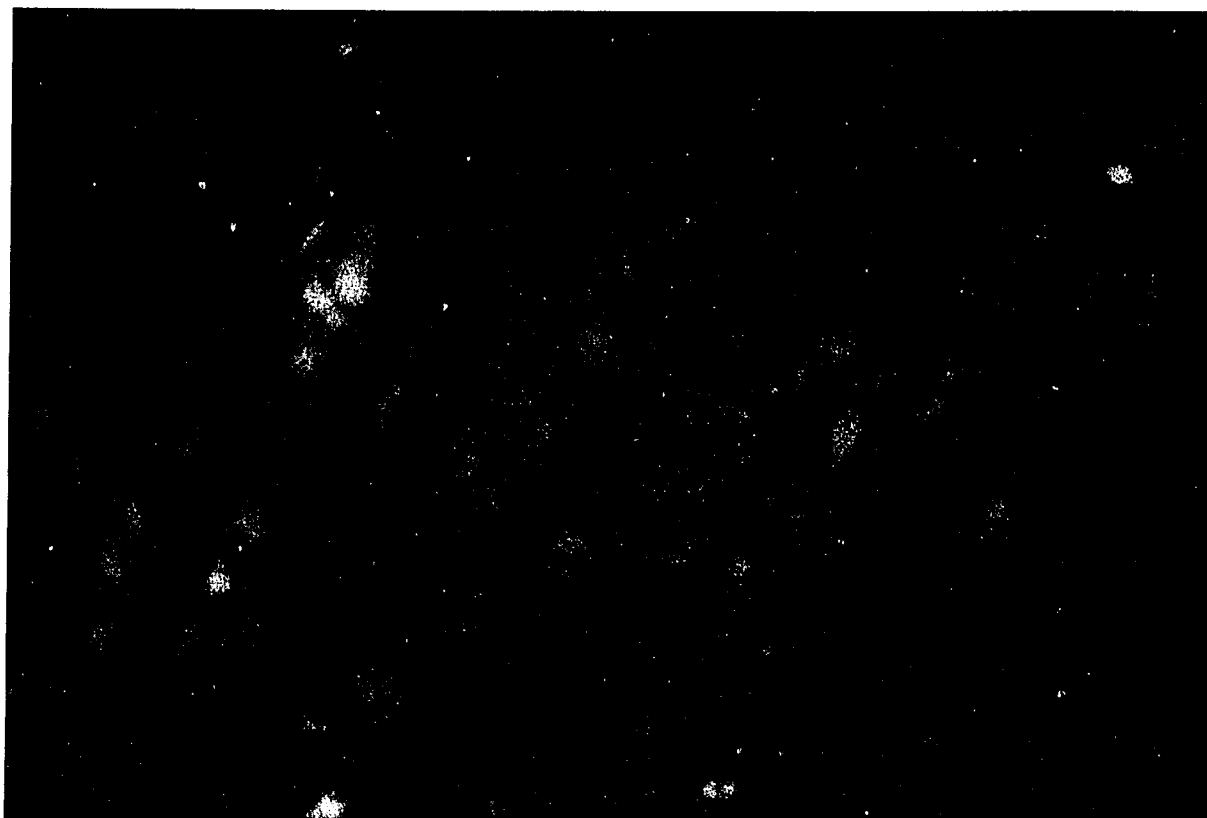


Figure 9.22: NA of an E20 GABA stained brainstem. As at E16, the nucleus is apparent by its relative lack of stain. Few interneurons can be seen within or around NA (e.g., arrow). The areas of lack of stain (1) correspond to ambigual motoneurons (see below). (GABA, 500x)

Figure 9.23: The above E20 NA fluoresced to reveal DAPI stained cell nuclei. As at E16, motoneurons comprise a considerably larger portion of the cell population than interneurons. Note that the unstained regions shown above correspond to the large cell nuclei of NA motoneurons (e.g., 1). (DAPI, 500x)

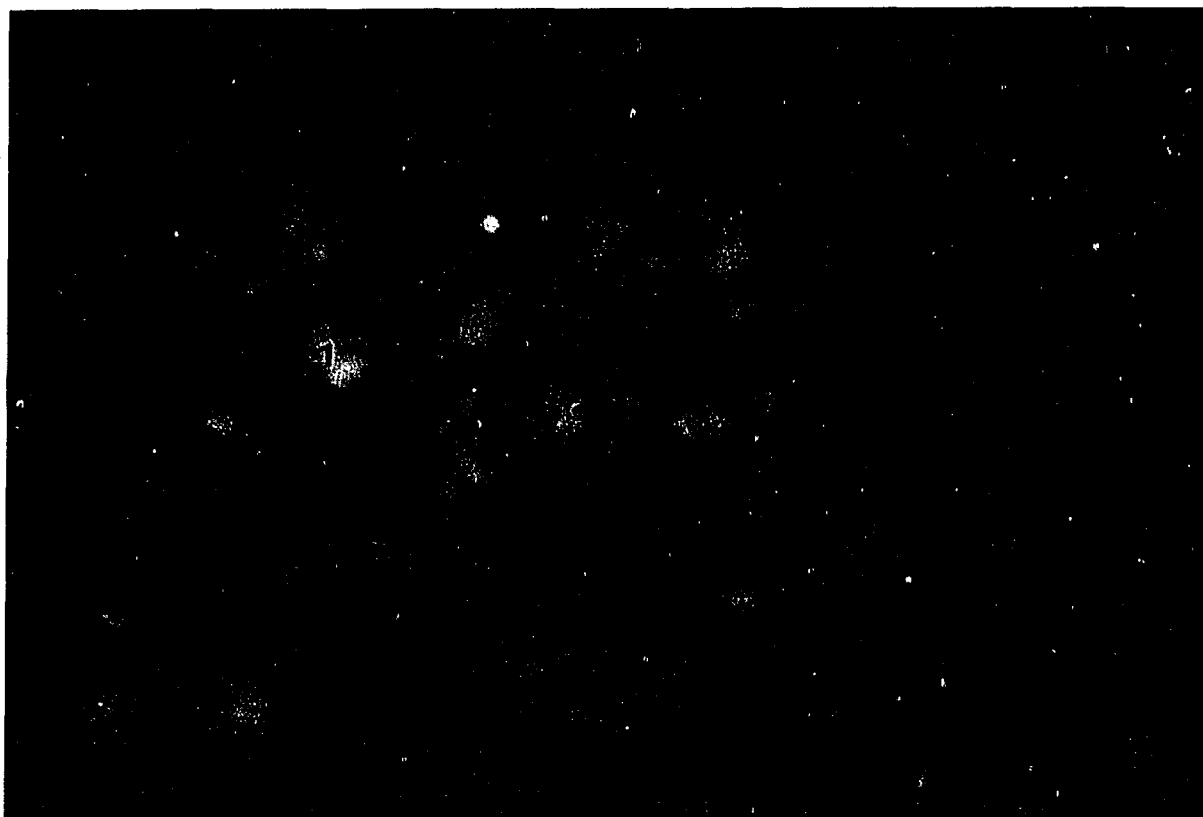
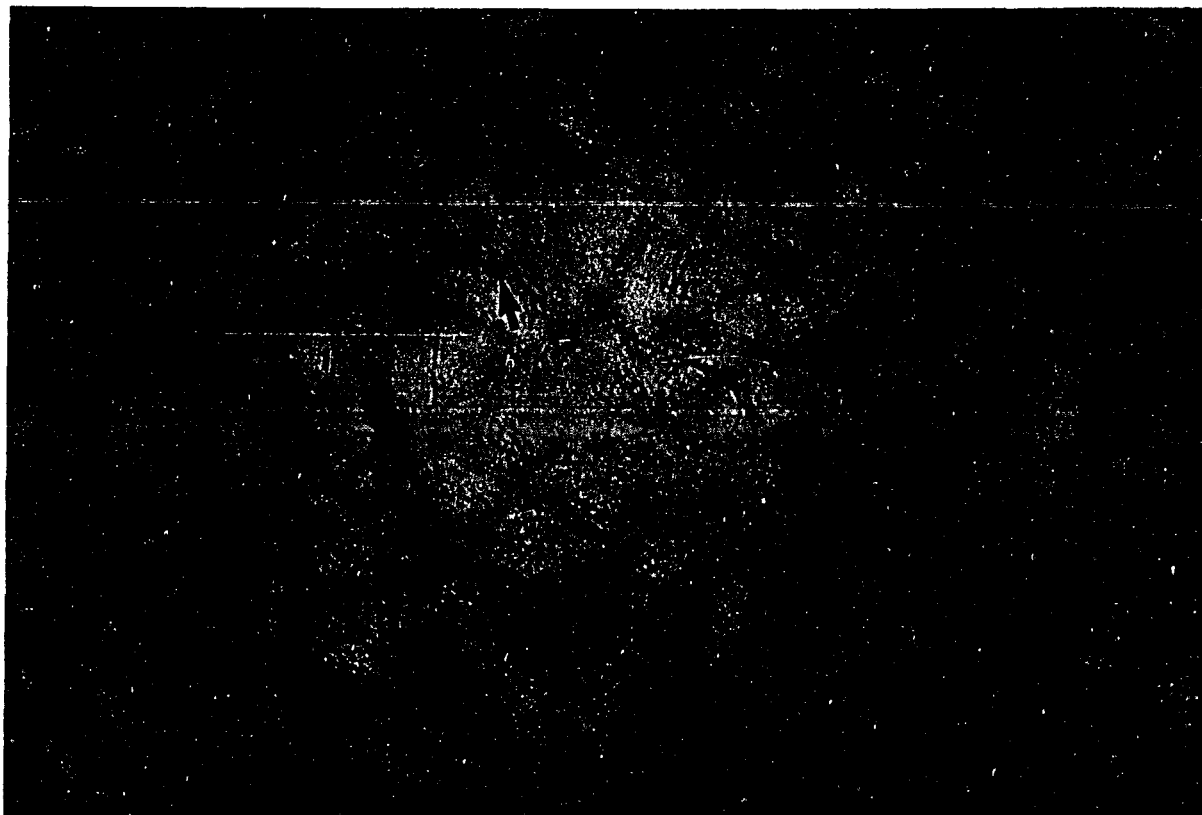
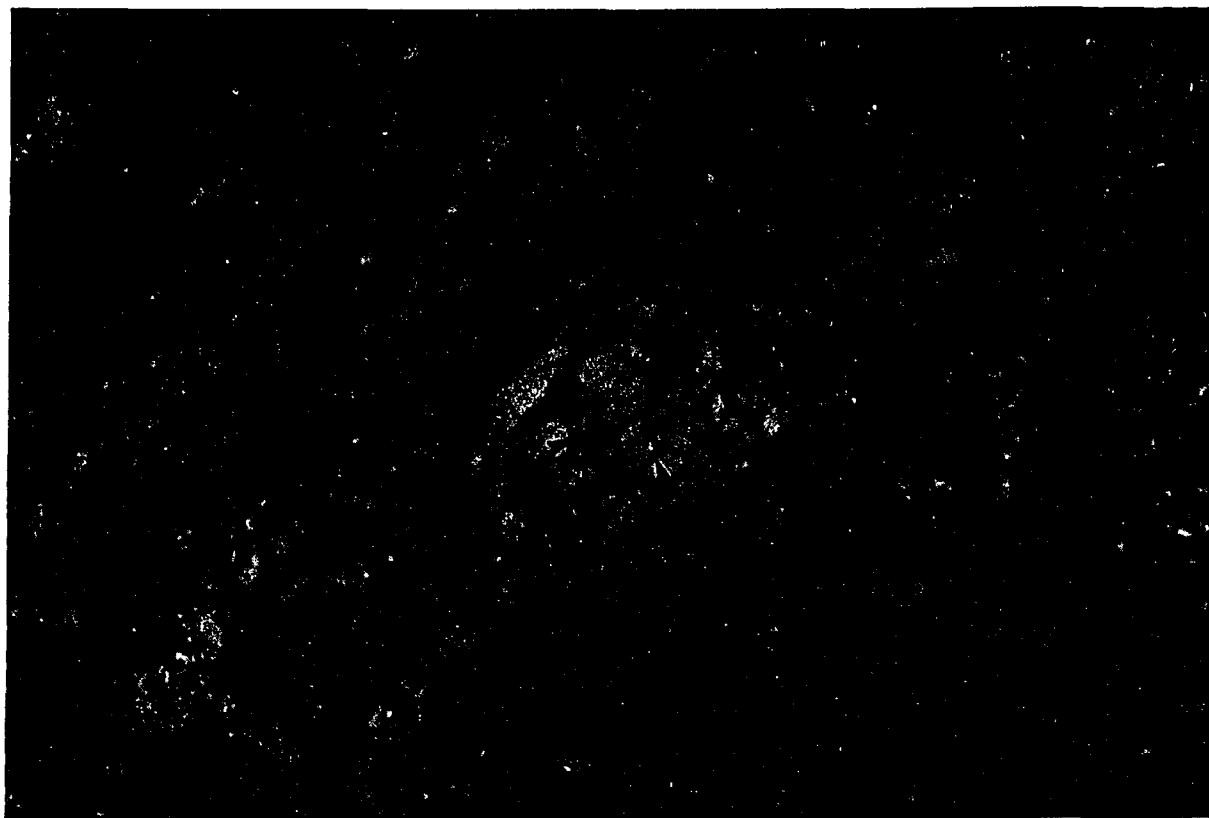


Figure 9.24: NA in an E16 GABA stained brainstem. Previous figures assessed the interneuron population in this nucleus but this figure focuses on the GABA-positive fibers. Relatively few fibers are seen at this stage (arrow head). (GABA, 320x)

Figure 9.25: NA in an E20 GABA stained brainstem. Although the nucleus is relatively unstained compared to the surrounding brainstem, many fibers can be seen to project to NA at this stage (arrow heads). This indicates the establishment of afferent neuronal input to NA motoneurons between E16 and E20. See text for further discussion. (GABA, 320x)



9.2.4 Discussion

Immunoreactivity for GABA was investigated in E16 and E20 rat brainstems to compare populations of motoneurons and interneurons during the period of MCD. The goal of this study was to indicate the effects of inadvertent inclusion of interneurons in cell counts on the observed course of MCD. In addition to staining for interneurons, the antibody defined the distribution of GABA-positive fibers within NA. Thus, the data may be analyzed with respect to: 1) relative interneuron numbers in the prenatal NA and the effect on cell counts; and 2) afferent neuronal input to NA motoneurons during the period of MCD.

9.2.4.1 Relative Interneuron Populations

GABA-positive neurons were found in the region of NA in E16 and E20 rat brainstems (figures 9.20, 9.22, 9.24, 9.25). They were few in number and were compared with the total neuronal population of NA by the use of DAPI nuclear staining. Such staining revealed many non-immunoreactive, large nucleated cells comprising NA (figures 9.21, 9.23). These cells were unable to be counted due to section thickness and density and overlap of DAPI stained nuclei. Based upon the DiI tracing studies and observations in H&E stained paraffin sections indicate that these cells are motoneurons. By using the motoneuron counts from the MCD study (see chapter 6), it can be determined that the interneuron population at both ages makes up a very small portion of the total neuronal population. This agrees with the findings of Mugnaini and Oertel (1985) in the adult rat in which they found interneurons in NA to comprise less than five percent of total cell number. Therefore, if large interneurons were inadvertently included in motoneuron counts it would not have significantly affected the number of estimated motoneurons nor the observed course of MCD.

9.2.4.2 GABAergic Innervation

While there were no significant changes in the number and distribution of GABA-positive cell bodies in NA, there appeared to be considerable changes in the distribution of immunoreactive fibers between E16 and E20. There were essentially no GABA-positive projections at E16, however, by E20 the nucleus was partly obscured by such fibers (compare figures 9.24 and 9.25). This period of increase in afferent neuronal input to NA coincides with the occurrence of MCD. As described in chapter 3, MCD is responsive, in part, to epigenetic signals from afferent neurons. Thus, the observed proliferation of afferent input to NA motoneurons at this time may play a modulatory role in the course of cell death.

Chapter 10

Hypoglossal Nucleus

Formation and GABAergic Innervation

10.1 Nucleus Formation

10.1.1 Introduction

The genesis of hypoglossal motoneurons and the mature location of the nucleus are in the same dorsomedial brainstem region. Thus, if cell migration played a major role in the formation of XII it would involve novel migratory pathways as yet undescribed. To investigate the role of cell migration in XII formation the neural tracer fast-DiI was used to determine the location of hypoglossal motoneurons at several developmental stages. Results of this study were also used to define a temporal relationship between migration of hypoglossal motoneurons and MCD in XII.

10.1.2 Experimental Design

This study investigated XII formation in E13, E15, and E17 rats. These ages were selected for two reasons: 1) these ages covered a period during which histological differentiation of XII was observed allowing the correlation of DiI and H&E studies; and 2) the same ages were studied in NA thus allowing direct comparison of temporal patterns of development between both URT motor nuclei. Unlike the neural tracing study in NA, E14 was not added after preliminary results indicated no significant changes between E13 and E15.

The methods used in DiI neural tracing studies were described in chapter 8. However, there were aspects of the tracing protocol tailored specifically for DiI application to the hypoglossal nerve. The inability to isolate the entire hypoglossal nerve required application of the dye to the nerve rootlets. This was accomplished by placing a small amount of DiI paste on the ventral surface of the lower medulla. This was found to be the only manner in which hypoglossal fibers could be traced. However, it was not specific so as to prevent diffusion of the dye rostrally and laterally to the vagus and glossopharyngeal nerves. In order to prevent this, the vagus and glossopharyngeal nerves

were clearly identified and sectioned as far from the brainstem as possible. This served to increase the distance between the applied DiI and the cut end of the unwanted fibers. Additionally, the trace was limited to 4 days which was long enough for specific DiI diffusion to XII but not enough time diffusion along the brainstem to the cut ends of the vagus and glossopharyngeal nerves. This maintained specificity of the trace to hypoglossal fibers and their motoneurons.

10.1.3 Results

The hypoglossal nerve, unlike the vagus and glossopharyngeal nerves, contains only one type of fiber. These are GSE fibers which project to the non-branchiomeric muscles of the tongue. Embryologically, GSE motoneurons in the brainstem arise from the medial part of the basal plate (see chapter 2). This is the same region of the brainstem where the mature XII is found. Thus, tracing studies of the developing hypoglossal nerve are unlikely to identify migrating neurons. They can, however, provide considerable information on the general organization of the nucleus at the onset of MCD.

10.1.3.1 Nucleus Formation: E13 through E17

As described in chapter 9, the earliest age in which DiI traces were able to be utilized was E13. Figure 10.1 shows a vibratome section of an E13 brainstem and indicates the location of XII along the fourth ventricle. Figures 10.2 and 10.3 show medium power views of an E13 brainstem by light and fluorescence microscopy. The hypoglossal nerve can be seen extending to the dorsomedial brainstem and defining a diffuse but coherent nucleus. That is, there are no apparent cells outside XII either migrating toward or migrating from the region. There is background signal along the ventral brainstem surface as the dye diffused through the connective tissue. In addition, some DiI diffused to the contralateral hypoglossal nerve thus indicating the right XII as well. In another

specimen that utilized less DiI, distinct fiber tracts projecting from XII can be seen coursing ventrally (figure 10.4).

By E15 the brainstem has increased dramatically in size which enables better visualization of XII and its respective fiber tracts. Figure 10.5 shows an unfluoresced section through the brainstem and indicates the location of XII. The result after fluorescence is shown in figure 10.6. Note the absence of signal elsewhere in the brainstem and the specificity of the tracer for the hypoglossal fibers and nucleus. On higher power separate fiber tracts can be seen projecting to a distinct nucleus (figure 10.7). No fibers are seen projecting to the contralateral nucleus nor to any cells outside XII. Focusing specifically on the nucleus, individual motoneurons and their axonal and dendritic processes can also be seen (figure 10.8). Note in particular the ventrolateral group of cells extending dendrites into the surrounding reticulum.

The results of E17 hypoglossal traces are shown in figures 10.9 through 10.12. There is little change in brainstem shape and in nucleus appearance between E15 and E17. As in E15 traces there is no signal outside the nucleus nor in the contralateral nucleus (figure 10.10). In addition, the fiber tracts are distinct in appearance and XII is well defined (figure 10.11). Also, apparent in the E17 XII is the small ventrolateral group of motoneurons with dendritic projections to the surrounding tissue. Figure 10.12 shows the results of a trace in which the DiI paste dissociated from the brainstem leaving only a small amount of tracer which defined individual motoneurons within a faintly traced XII. Note the axon of the labeled neuron projecting toward the hypoglossal nerve and the extensive arborization of the dendrites both intrinsic and extrinsic to XII.

Figure 10.1: Vibratome section through an E13 brainstem. Fast-DiI was placed on the hypoglossal nerve just after emerging from the brainstem (arrow). The fourth ventricle is indicated for orientation (v). For further orientation see figures 2.2 and 9.1, and chapter 4. (unstained, 80x)

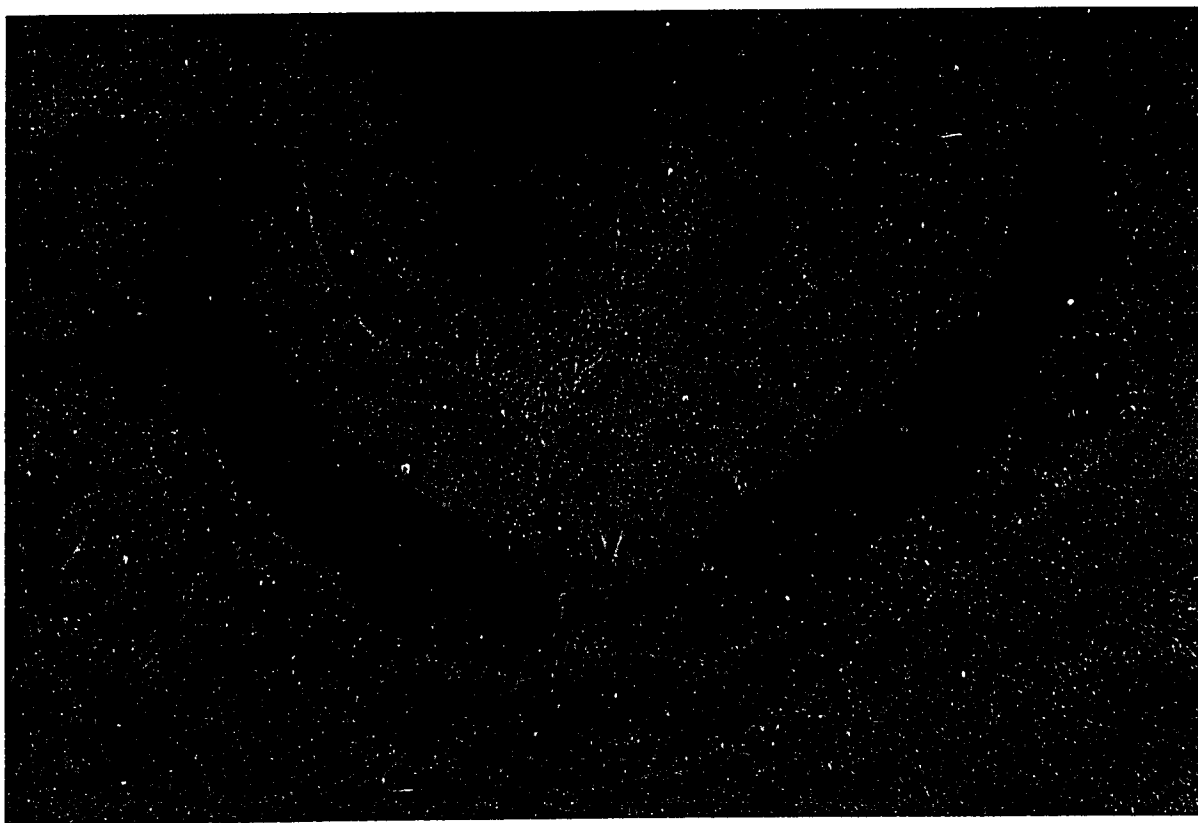


Figure 10.2: Closer view of middle region of E13 brainstem seen in figure 10.1. The left and right hypoglossal nerves where fast-DiI was placed are indicated (arrows) as is the mature location of XII (arrow heads). For further orientation see the previous figure. (unstained, 125x)

Figure 10.3: Above E13 brainstem fluoresced for fast-DiI. Both left and right hypoglossal nerves were traced to XII (arrow indicates right XII). There was some non-specific diffusion of dye in the ventral connective tissue but the specific tracing into the brainstem illuminated only XII. (fast-DiI, 125x)

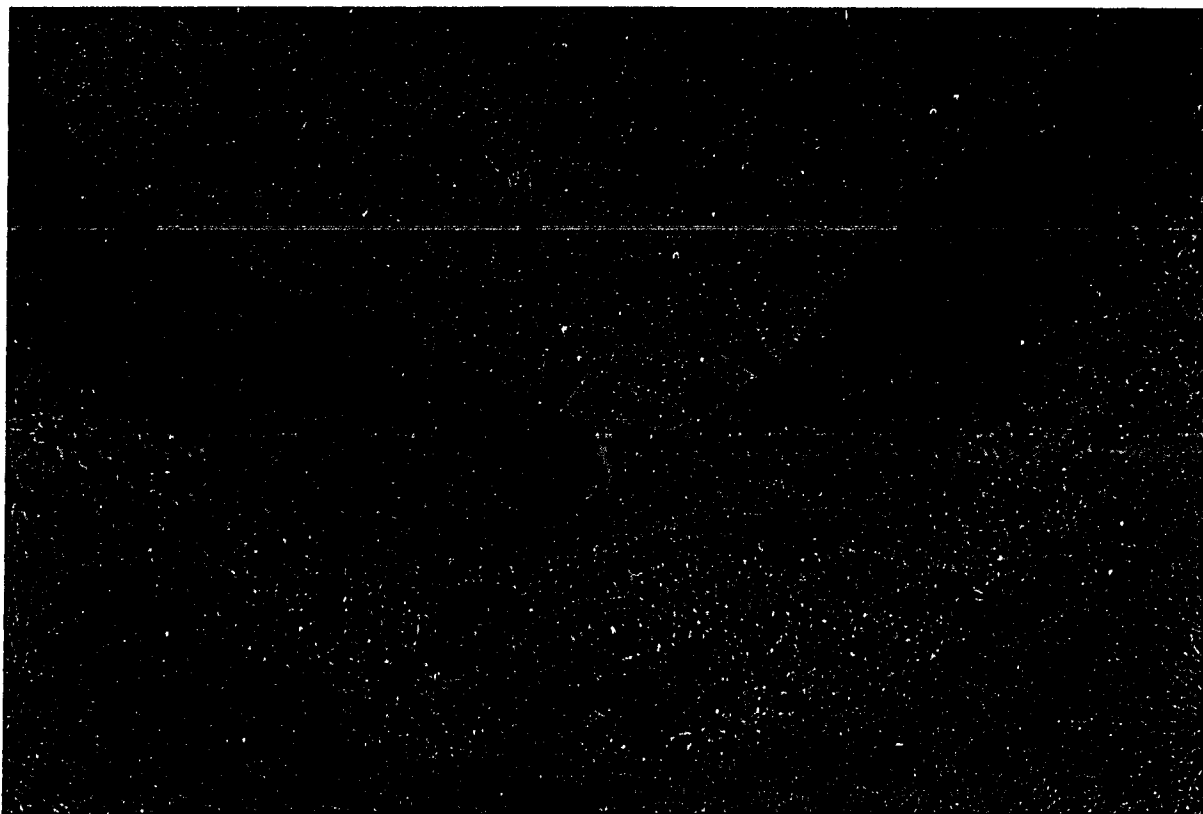


Figure 10.4: Closer view of the left E13 XII from the previous figures. The hypoglossal fibers can be clearly seen within the brainstem (arrow) extending dorsally to XII. No other fibers were traced to any cells outside XII. The structure to the right of XII is a blood vessel. (fast-DiI, 250x)



Figure 10.5: Transverse section through an E15 brainstem at the level of XII. Indicated is the left hypoglossal nerve where fast-DiI was placed (arrow). Also indicated is the location of XII (arrow head). For further orientation see figures 7.6 and 7.8 which show the E15 XII in histologic preparations. (unstained, 80x)

Figure 10.6: Above E15 brainstem section fluoresced for fast-DiI. Fibers can be seen to extend from the peripheral nerve at the bottom of the figure to a clearly defined XII near the top (corresponds to above labeled figure). No signal is seen outside the nucleus indicating that no hypoglossal neurons are migrating to XII during this period. (fast-DiI, 80x)

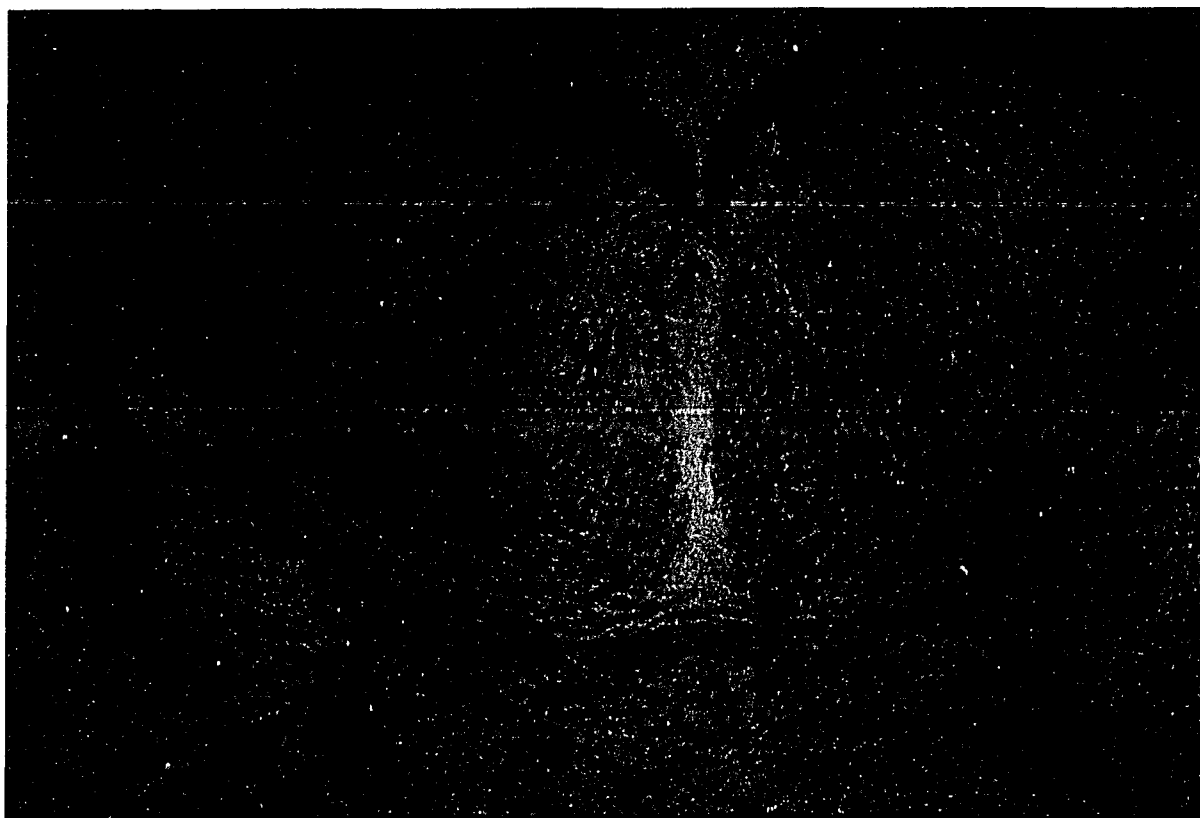


Figure 10.7: Closer view of the E15 XII seen in figure 10.6. Bottom of figure is right side of page (i.e., rotate 90 degrees). Note the small ventrolateral subdivision of the nucleus (arrow). This indicates that by E15 XII has begun to differentiate into discrete neuronal subpopulations. See text for further discussion. (fast-DiI, 160x)

Figure 10.8: E15 XII from another specimen indicating the ventrolateral subdivision (as in above figure). Bottom of figure is right side of page (i.e., rotate 90 degrees). This subdivision has been shown to correspond to innervation of the geniohyoid muscle (see text). (fast-DiI, 250x)

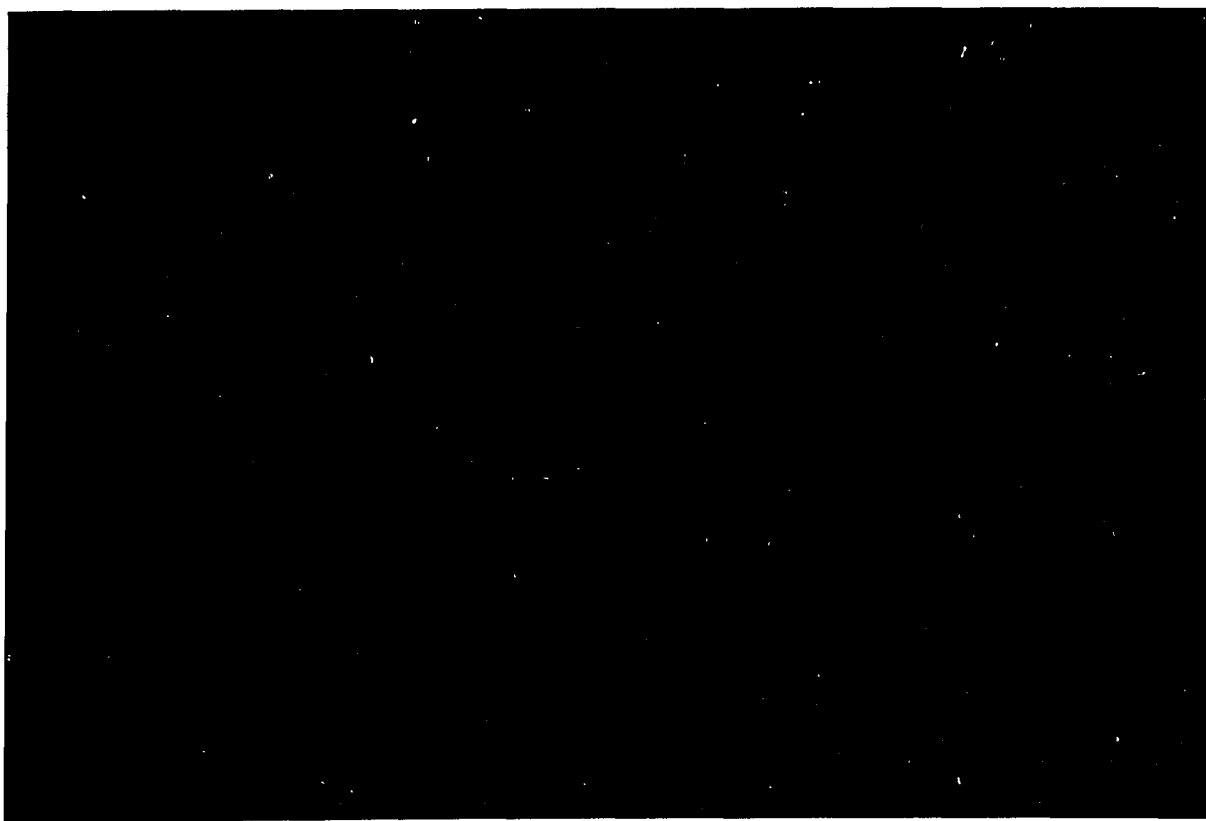
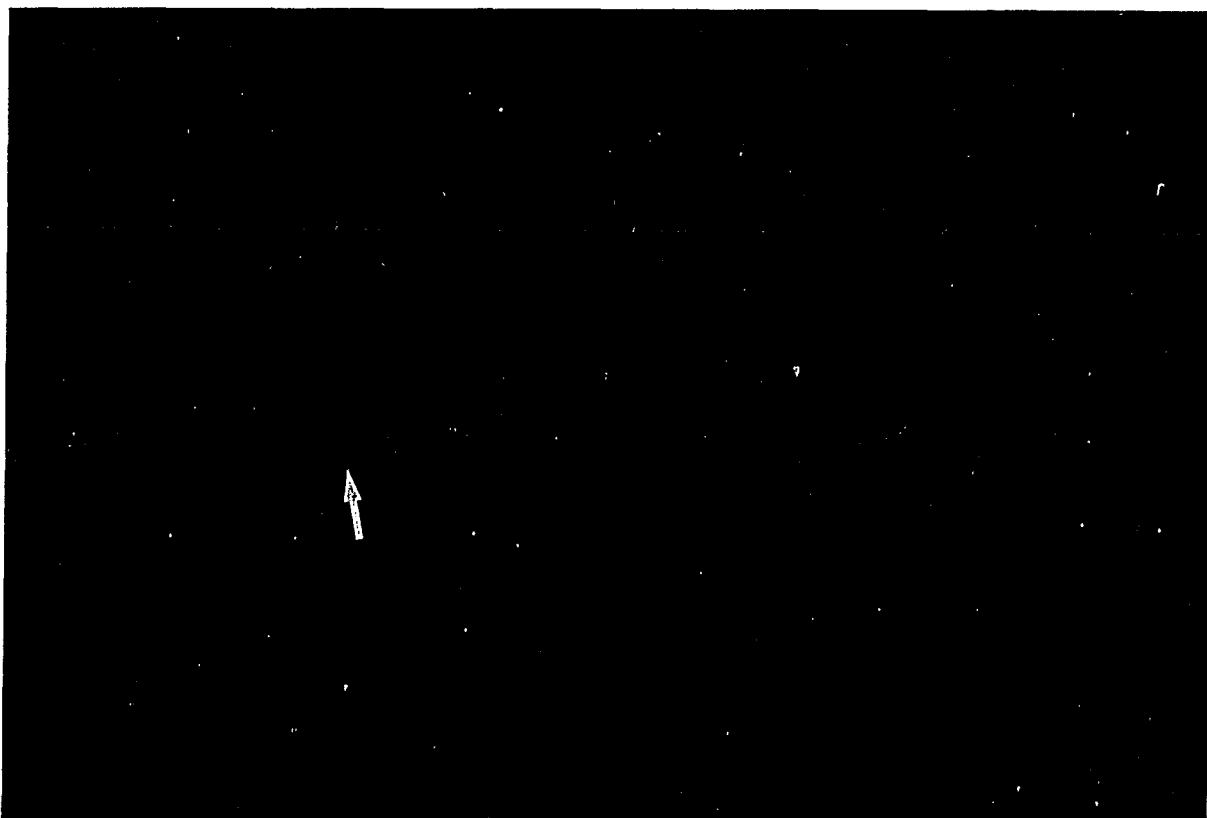


Figure 10.9: Transverse section through an E17 brainstem at the level of XII. This section is for orienting the neural trace shown below. Indicated is the nerve where fast-DiI was placed (arrow) and the dorsomedial location of XII (arrow head). (unstained, 50x)

Figure 10.10: Above E17 brainstem section fluoresced for fast-DiI. Hypoglossal nerve fibers can be seen extending from the bottom of the figure where dye was placed dorsally to XII (corresponds to above figure). No signal is seen outside the nucleus. (fast-DiI, 50x)

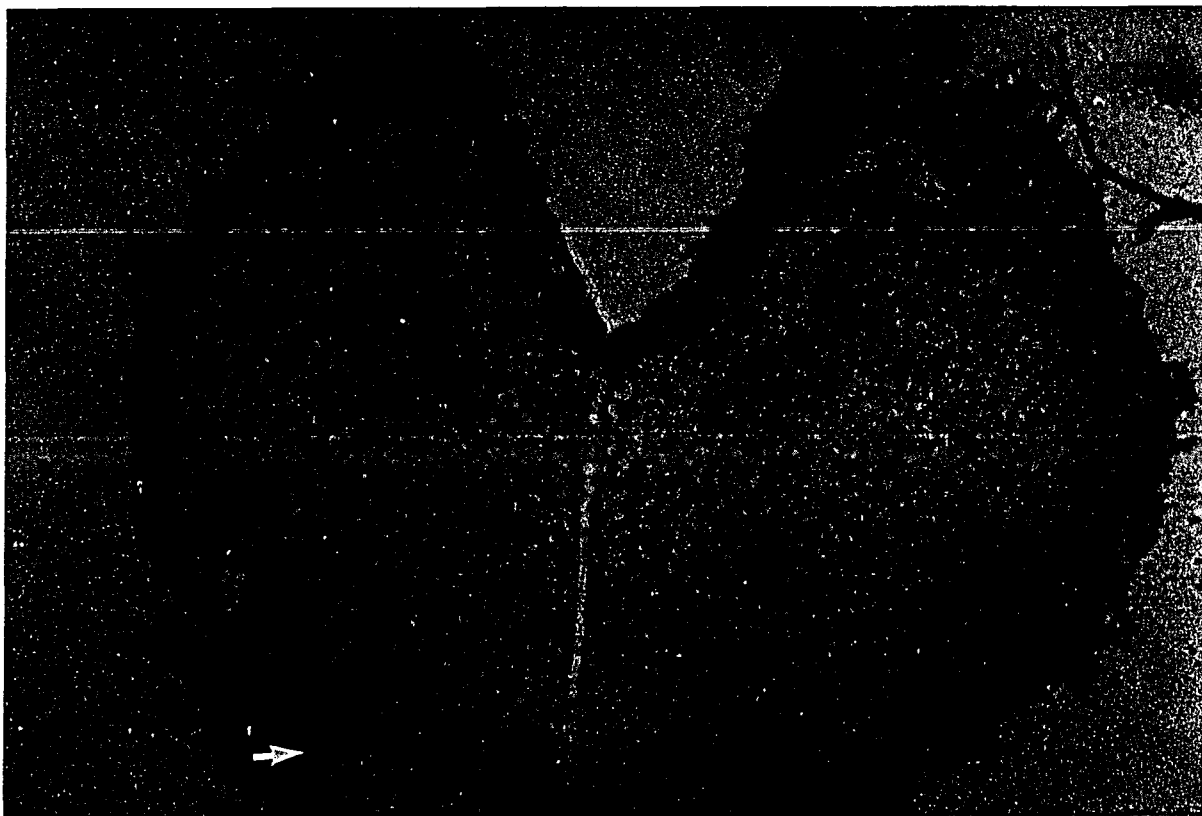


Figure 10.11: Closer view of XII in an E17 DiI-traced brainstem. Bottom of figure is right side of page (i.e., rotate 90 degrees). Note the presence of the ventrolateral subdivision as seen at E15 (arrow). Dendritic fibers can be seen extending into the reticular formation to the left of the nucleus above and below the arrow. (fast-DiI, 160x)

Figure 10.12: E17 XII inadvertently stained lightly so that one neuron was prominently illuminated. Bottom of figure is right side of page (i.e., rotate 90 degrees). This neuron is in the geniohyoid subdivision (see text). An axon can be seen clearly projecting from this motoneuron to the hypoglossal nerve (arrow). This distribution is contrary to traditional teachings in anatomy as to the origin of geniohyoid motoneurons (see discussion in text). On the top of the neuron (opposite from axon) dendritic fibers can be seen projecting into XII (toward the right) and into the surrounding reticular formation (toward the left). (fast-DiI, 250x)



10.1.4 Discussion

DiI was used to trace hypoglossal nerve fibers back to XII in prenatal rats between ages E13 and E17. This study was directed at elucidating the temporal relationship between nucleus formation and MCD. In addition, this study sought to determine whether XII was fully formed at E16, the first age at which motoneuron counts were made.

E13 was the earliest age studied and, although the neural traces were not as distinct as those at later stages, they indicated that hypoglossal motoneurons were present in the region of XII. Moreover, no motoneurons were found outside XII indicating that, unlike NA, XII undergoes little if any cellular migration at this time. Studies of neurogenesis indicate that hypoglossal motoneurons are generated around E10 and E11 (Altman and Bayer, 1980; Phelps et.al., 1990) and that they arise in the same dorsomedial brainstem region where the mature nucleus is found (Kimmel, 1940; Windle, 1970; Brown, 1990). Thus, any migration that occurs would be immediately prior to E13 and not involve pathways outside the vicinity of the dorsomedial brainstem.

The E15 brainstems were larger and allowed for better specificity in the trace with less background fluorescence from the ventral connective tissue (compare figures 10.3 and 10.6). While there was little change in the gross pattern of nerve fiber projections and nucleus location, XII itself appeared more organized by E15. This was evident by the emergence of a clearly defined ventrolateral group of neurons, slightly detached from the body of the nucleus. Neural tracing studies in adult animals indicates that this group may represent motoneurons to the geniohyoid muscle (Uemura et.al., 1979; Chibuzo and Cummings, 1982; Kitamura et.al., 1983; Kitamura et.al., 1985; Sokoloff and Deacon, 1992). Thus, as seen with DiI, the E15 XII appears to be fully formed which agrees with the observation of a fully distinct nucleus at E15 in H&E stained tissue (see chapter 7).

There was little change in appearance of XII between E15 and E17 in the DiI traced brainstems. A distinct ventrolateral group of motoneurons continued to be observed as

were extensive dendritic arbors projecting into XII and the surrounding reticular formation (figures 10.11 and 10.12). In fact, the only noticeable change between E15 and E17 was an increase in size of the nucleus (compare figures 10.8 and 10.12 which are at the same magnification). Thus at E16, the first age at which MCD was assessed, XII is fully formed and well organized with no new motoneurons migrating into the region. As such, motoneurons counts at this stage include all hypoglossal motoneurons and do not underestimate the total number of cells. Furthermore, because migration of SVE motoneurons to NA is complete by E16 (see chapter 9), XII contains only GSE motoneurons and no late migrating SVE motoneurons were inadvertently included in the cell counts.

10.2 GABAergic Innervation

10.2.1 Introduction

The effect of large interneuron populations on the assessment of MCD was of particular concern as recent reports demonstrated such cells within XII of monkeys and rats (see chapter 2). As described in chapter 9, antibodies to GABA were used to identify interneurons at key developmental stages in the rat NA. These same specimens were used to explore the development of GABAergic innervation in the rat XII as well.

10.2.2 Experimental Design

Rat brainstems at developmental ages E16 and E20 were immunostained for GABA following the protocol outlined in chapters 8 and 9 and appendix E. These ages enabled GABAergic innervation within XII, and in comparison to NA, to be assessed throughout the period of MCD. Unlike the DiI studies in which the URT motor nuclei had to be studied in separate brainstems, the GABA study enabled the examination of NA and XII in the same specimens. Accordingly, sample size is the same as discussed in chapter 9.

10.2.3 Results

The GABAergic staining of XII at E16 and E20 was similar to that seen in NA. As such, in addition to being able to assess relative interneuron populations, the establishment of GABAergic fibers in XII was also observed. Thus, the results of this study will be presented in two parts: 1) the relative population of interneurons to motoneurons in NA during the period of MCD; and 2) the establishment of GABAergic innervation patterns in the developing NA between E16 and E20.

10.2.3.1 Relative Interneuron Populations

A control section at the level of XII (caudal to obex) is shown in figure 10.13 with the location of XII indicated. The homogeneous appearance of this control section was in stark contrast to the findings in experimental brainstems. In E16 brainstems, XII was particularly conspicuous by its lack of stain against the surrounding highly reactive tissue (figures 10.14 and 10.15). In addition, little staining was found in the neighboring dX which like XII contains primarily efferent cholinergic neurons. Although less dramatic at E20, XII also stained lighter than the surrounding tissue and was characterized by a "honeycomb-like" appearance with many areas of non-reactivity surrounded by positive staining fibers (figures 10.16 and 10.21).

Examination of the E16 XII under higher magnification demonstrated a relative lack of staining for GABA-positive neurons although a few immunoreactive fibers were observed projecting into the nucleus (figure 10.17). To indicate the presence of non-staining motoneurons the section was stained with DAPI and fluoresced. Many large nuclei, although faintly stained, were observed in XII, densely packed and often overlapping one another (figure 10.18). In some GABA stained sections several interneurons were observed within XII but these were far outnumbered by the non-immunoreactive DAPI stained motoneurons (Figures 10.19 and 10.20). Note that the interneurons tended to cluster along the lateral border of the nucleus midway between its ventral and dorsal divisions.

In the E20 XII it was difficult to see the GABA-positive interneurons due to the abundance of immunoreactive fibers (figure 10.21). However, some cells were observed in the middle of the nucleus along the lateral border as was found in the E16 brainstems. In order to determine the relative number of interneurons to motoneurons these sections were also stained with DAPI. The larger size of the brainstem, and the fewer motoneurons due to MCD, enabled the observation of individual cell nuclei which were found in the non-stained areas between the immunoreactive fibers (figure 10.22). Thus,

at E16 and E20, motoneuron populations are substantially greater than the populations of interneurons in XII.

10.2.3.2 GABAergic Innervation

The difficulty in clearly demonstrating interneurons in the E20 XII stemmed from the abundance of GABA-positive fibers throughout the nucleus. This was not a problem in E16 specimens as there were relatively few reactive structures present in XII.

Comparing XII at E16 and E20 demonstrated the dramatic increase in GABAergic innervation during this gestational period (figures 10.15 and 10.16). These fibers, like those in NA, surrounded areas of non-reactivity shown by DAPI to correspond to hypoglossal motoneurons. Furthermore, on higher magnification these fibers appeared to be establishing synaptic contact within XII (figure 10.23).

10.2.4 Discussion

Developing brainstem motor nuclei were investigated for the presence of interneurons as recent reports have indicated that such cells may be found in XII (Cooper 1981b; Boone and Aldes, 1984, Mugnaini and Oertel, 1985; Takasu and Hashimoto, 1988). The presence of large numbers of interneurons in XII could have effected estimates of total cell numbers and led to an incorrect assessment of MCD. To assess the proportions of these cells, an antibody directed at the interneuron neurotransmitter, GABA, was used to stain E16 and E20 brainstems.

While many sections through the E16 brainstem showed no GABA-positive cells in XII (figure 10.17), some sections revealed a small number of such neurons (figure 10.19). The use of DAPI staining showed that interneurons were far outnumbered by motoneurons although direct counts of these thick sections was not possible (figures 10.18 and 10.20). These data, as those in NA, suggest that hypoglossal interneurons represent a small portion of the total neuron population. This finding agrees with those

of Mugnaini and Oertel (1985) who demonstrated very few GABA-positive neurons in the adult rat XII.

Interneurons in the E20 XII were not readily apparent, being partly obscured by the large number of immunoreactive fibers. Closer inspection, however, demonstrated a small number of interneurons within XII. The use of DAPI also showed that interneurons made up a minor part of the total neuronal pool. As such, if interneurons were included in the cell counts they would have no significant effect on the estimated number of motoneurons nor on the observed course of MCD.

Interestingly, in both ages the interneurons were found to cluster along the lateral border of the nucleus lying roughly midway between dorsal and ventral divisions (figures 10.20 and 20.22). This may have functional significance as these interneurons are in position to receive afferent input from neighboring swallowing and respiratory centers. In addition, interneuron position within the nucleus places them between the retrusor and protrusor divisions of XII and they may therefore modulate activity between the two functional groups.

As discussed in chapter 3, the course of MCD is dictated, in part, by epigenetic signals from afferent neurons. This study has demonstrated that interneurons are present in and around XII by E16, the first age at which MCD was assessed. Furthermore, between E16 and E20, afferent input to hypoglossal motoneurons appeared to be developing as the number of immunoreactive fibers and terminals dramatically increased between these stages (figures 10.15, 10.16, 10.23). These ages correspond to the period of MCD in XII which was found to occur between E16 and E19 (see chapter 7). Thus, afferent input to hypoglossal motoneurons may play an important role in defining the course of MCD. Furthermore, cell death may ensure that the afferent projections are specific by eliminating those motoneurons receiving improper input. This co-temporaneous maturation of afferent and efferent neuronal populations may establish a coordinated developmental sequence critical to the formation of postnatal URT control.

Figure 10.13: Control section through an E20 brainstem at the level of XII. Primary antibody for GABA was omitted from the incubate and replaced with normal serum. Note that the brainstem appears uniformly light brown with no distinction of different regions apparent. (GABA control, 50x)

Figure 10.14: Transverse section through E16 brainstem stained for GABA. Note that XII (arrow shows left nucleus), lying along the fourth ventricle, is conspicuous by its relative lack of staining. For further orientation see figure 2.2 and chapter 4. (GABA, 50x)

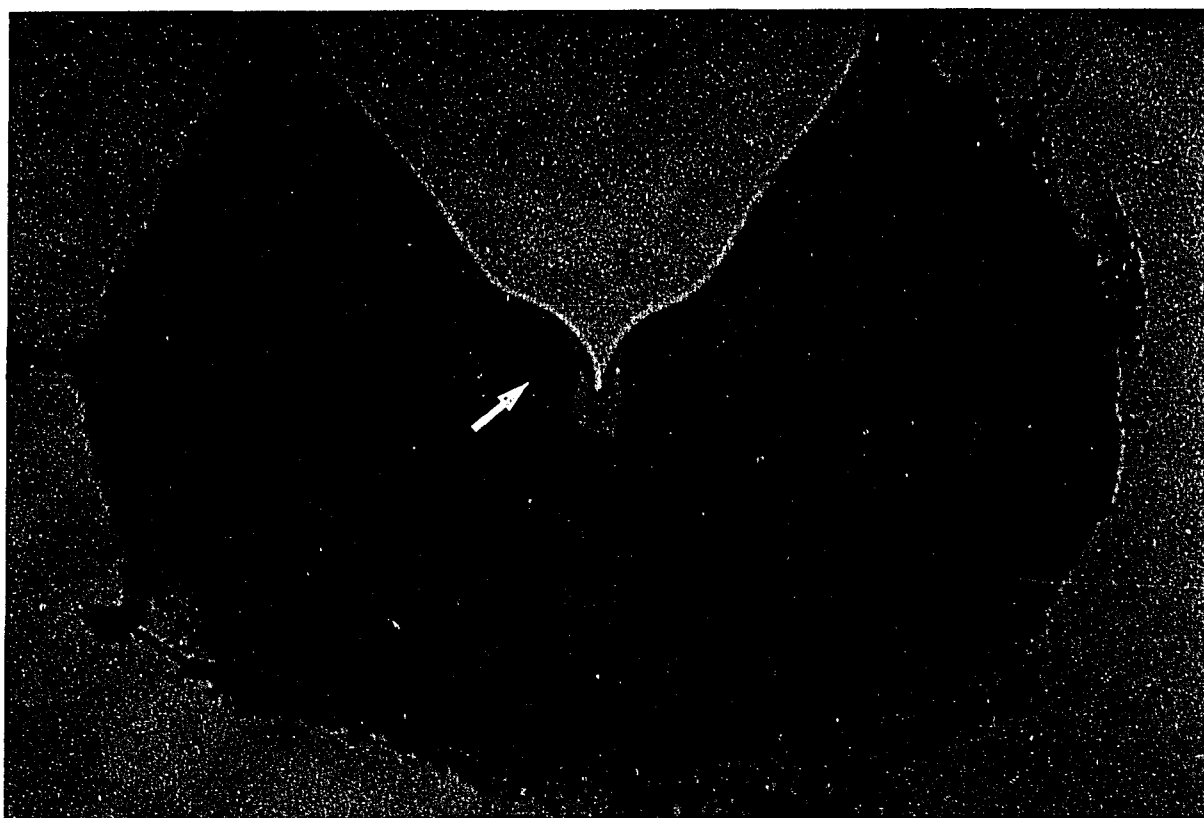
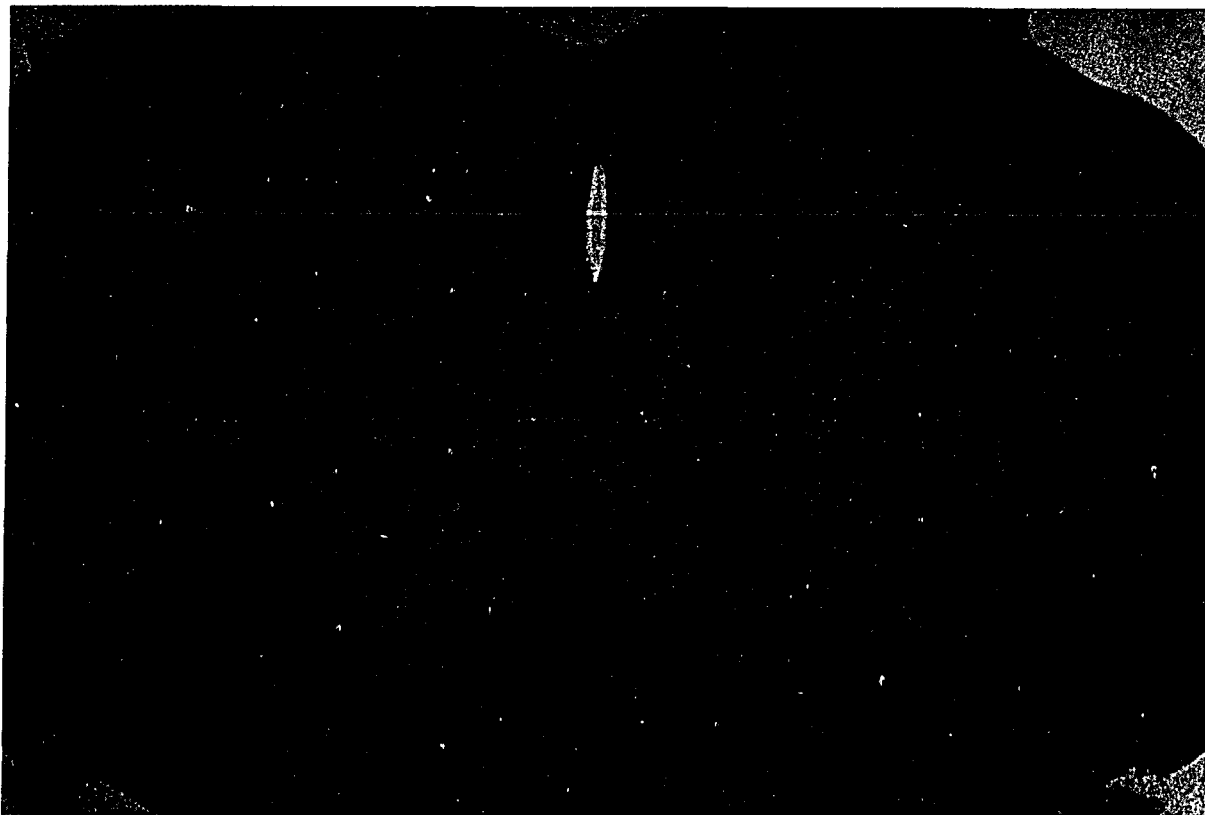


Figure 10.15: Left and right XII in E16 brainstem stained for GABA. The nuclei are conspicuous by their relative lack of staining as compared to the surrounding tissue. Although largely unstained, some fibers can be seen projecting into XII (arrow head). (GABA, 250x)

Figure 10.16: Left and right XII in E20 brainstem stained for GABA. XII is defined by a "honeycomb" pattern of GABA-positive fibers throughout its extent (e.g., arrow heads). This staining pattern indicates that between E16 (see above) and E20, GABA-positive afferent projections to NA motoneurons are established. The implications of this finding for the course of MCD are discussed in the text. (GABA, 250x)

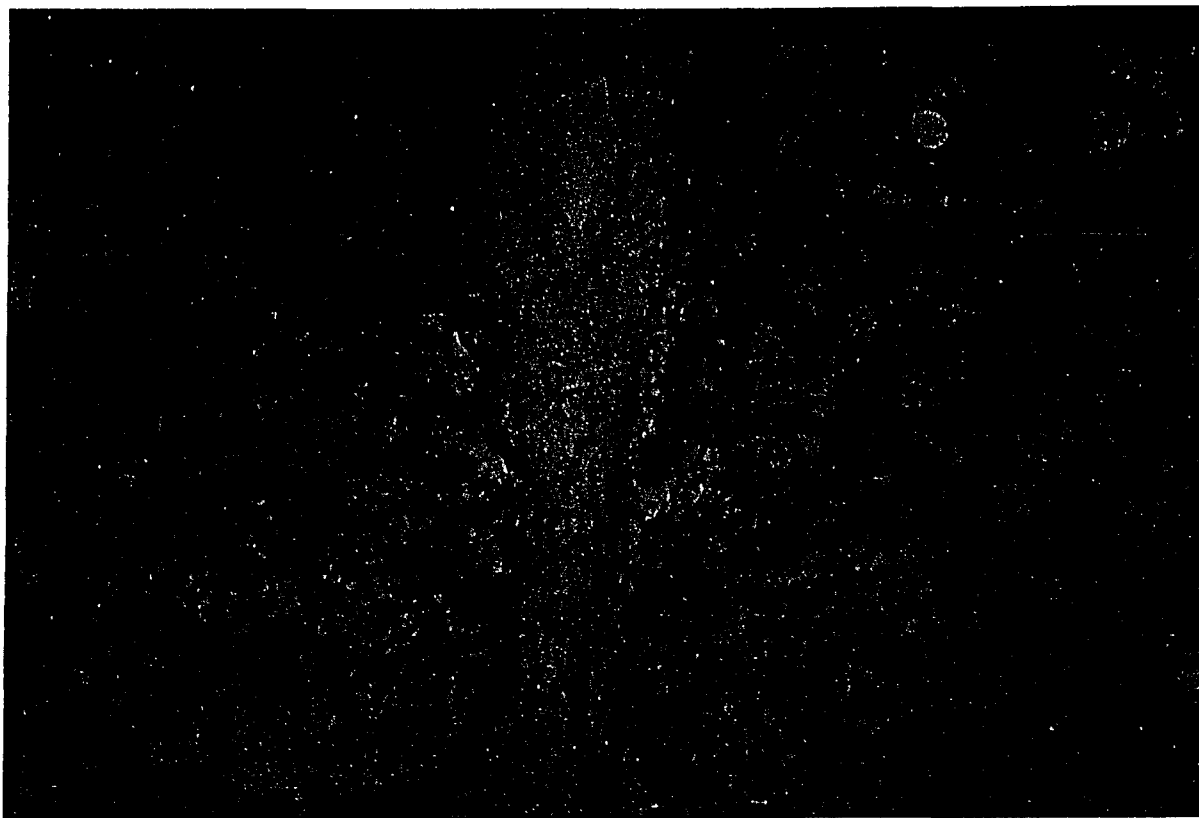
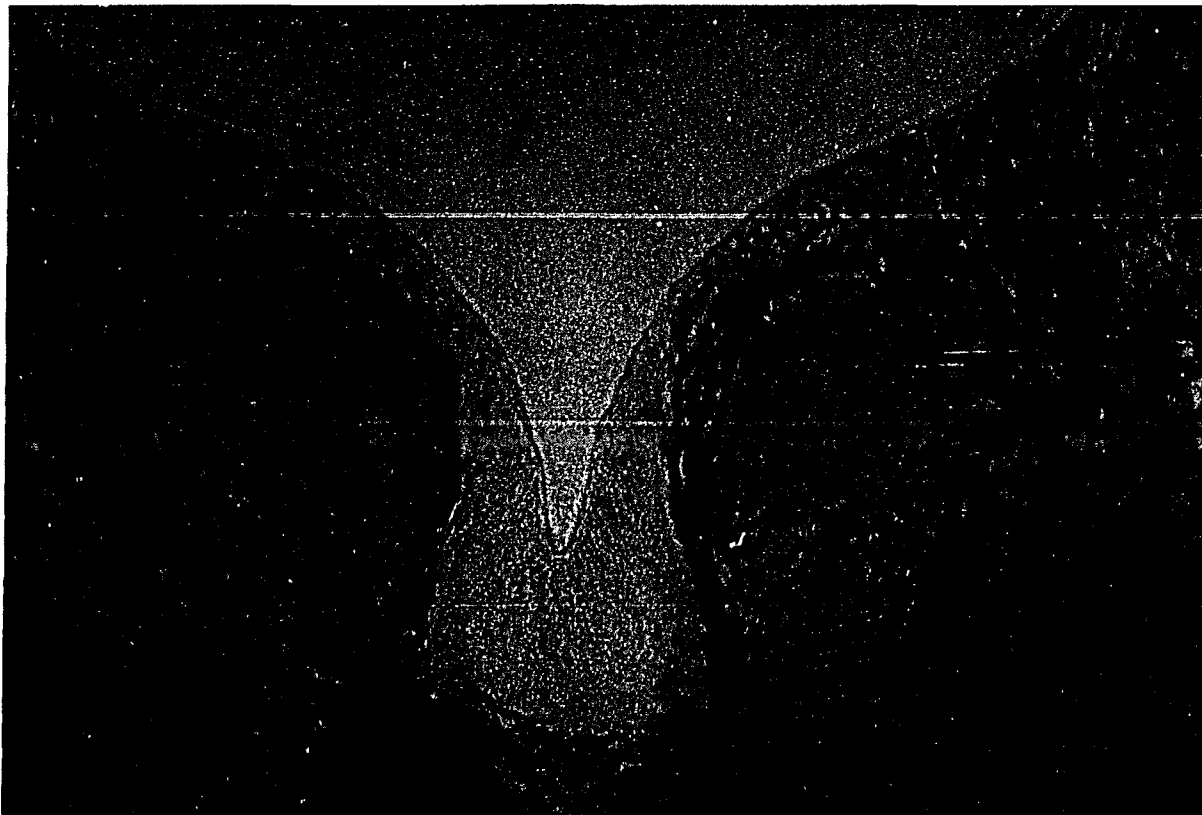


Figure 10.17: Closer view of E16 GABA stained XII shown in previous figures. This figure shows the relative lack of interneurons within XII. GABA-positive interneurons are seen, however, along the periphery of the nucleus (e.g., arrow). (GABA, 320x)

Figure 10.18: Above E16 section fluoresced to show DAPI stained cell nuclei in XII. Pale blue, oval cell nuclei are seen throughout XII (boundaries of XII correspond to above figure). This indicates that the vast majority of cells in XII are hypoglossal motoneurons. See text for further discussion. (DAPI, 320x)

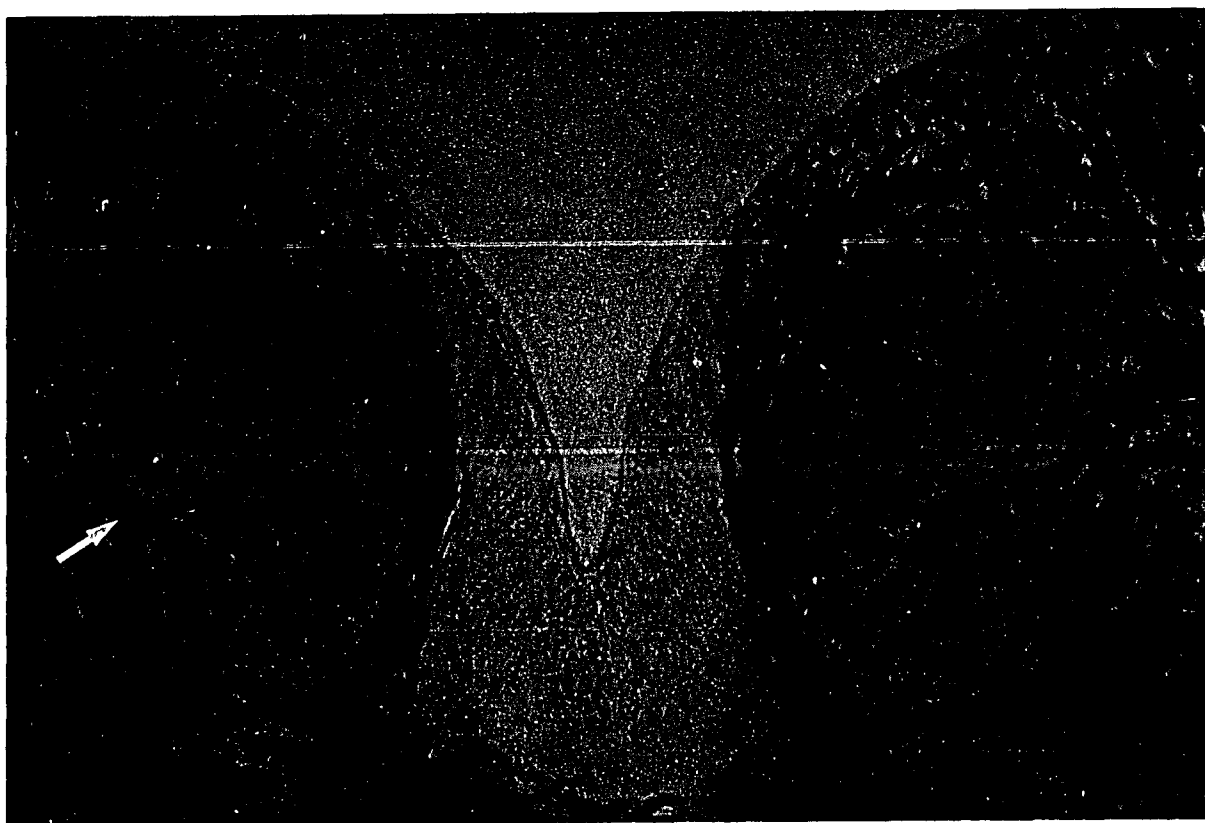


Figure 10.19: XII in an E16 brainstem stained with GABA. In this section several GABA-positive interneurons were found within XII. These projected in the midline of the nucleus. To assess the relative population of these interneurons to motoneurons, the section was stained with DAPI (see below). (GABA, 320x)

Figure 10.20: Above E16 XII fluoresced to show DAPI stained cell nuclei. There are a considerably larger number of small, pale blue stained motoneuron nuclei than GABA stained interneurons. This figure corresponds to the above figure for orientation and location of interneurons and boundaries of XII. (DAPI, 320x)

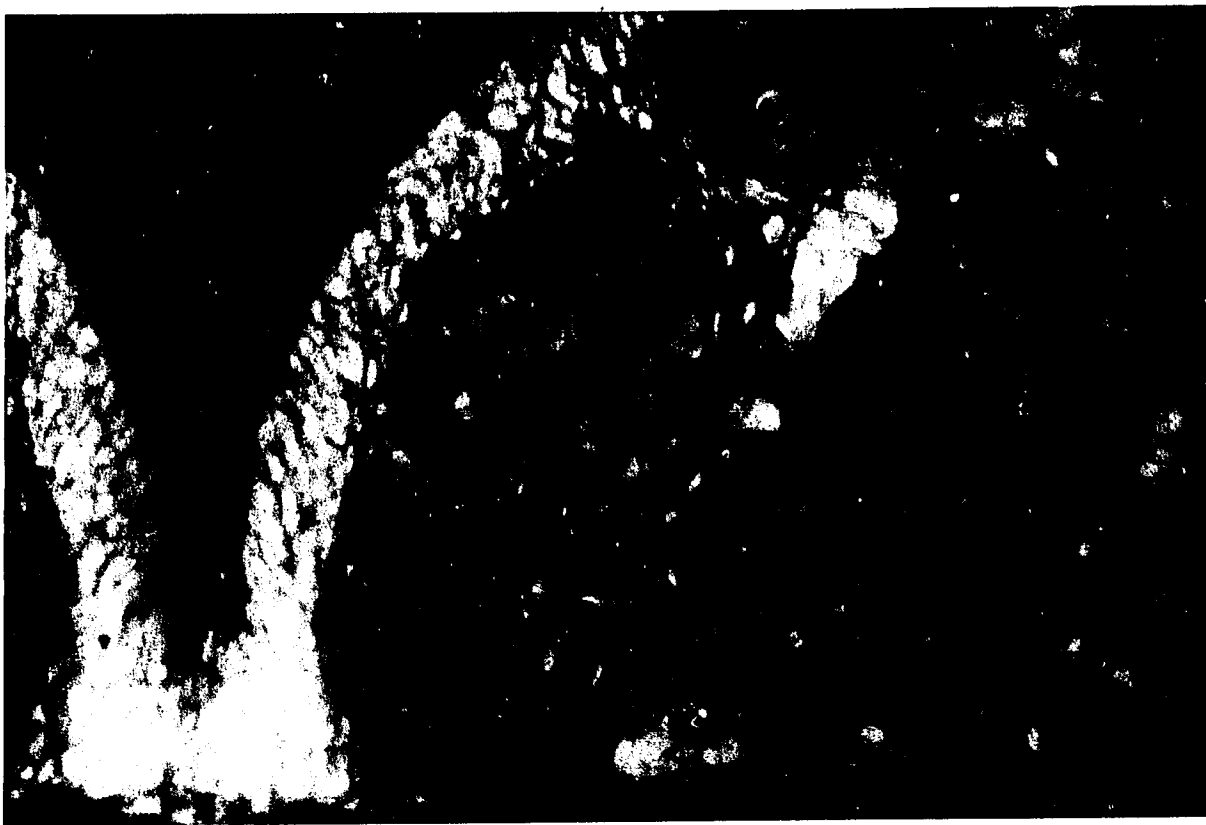
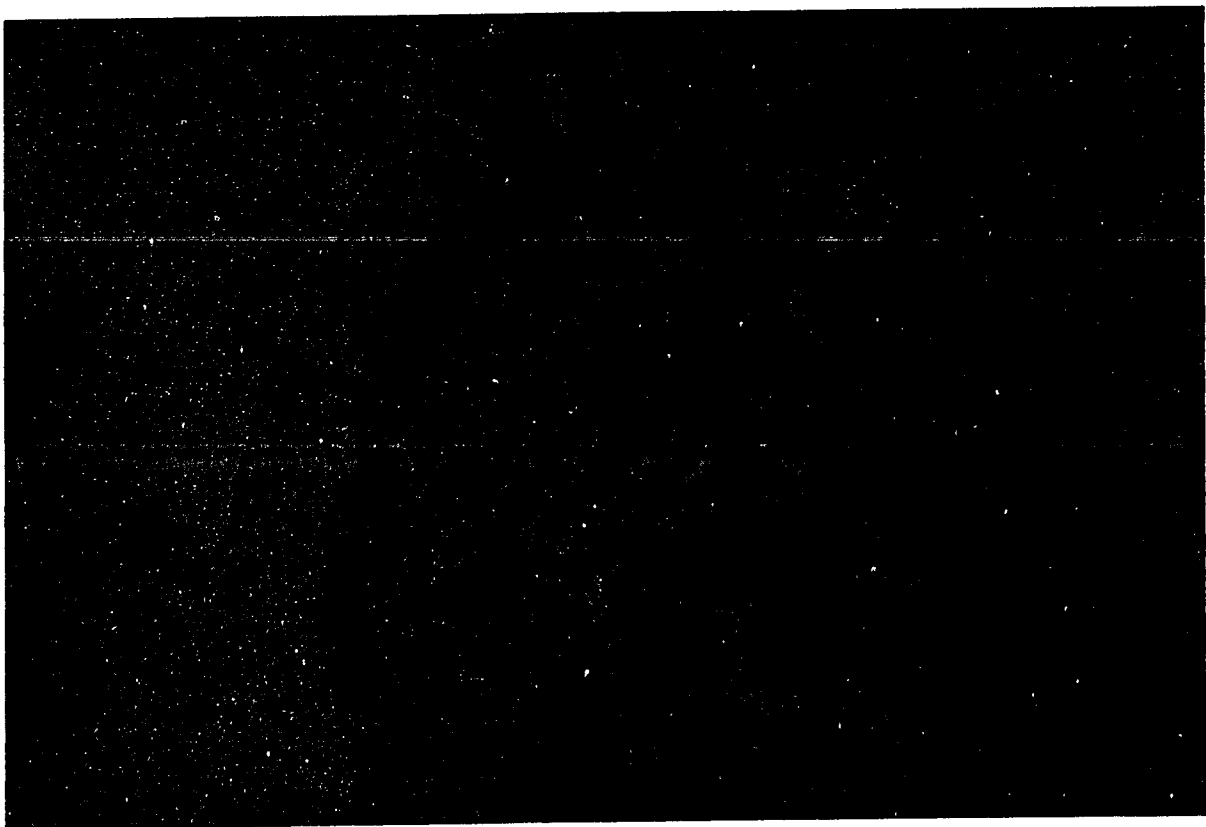


Figure 10.21: Left and right E20 XII stained for GABA. Although obscured by the many GABA-positive fibers (see figure 10.16), a few interneurons were present within the E20 XII (e.g., arrow). As in the E16 XII, these interneurons projected in the midline of the nucleus. In order to assess relative motoneuron populations the section was stained with DAPI (see below). The spaces between the "honey-combed" fibers can be seen to correspond to nuclei of hypoglossal motoneurons (note correspondence of space marked "1" and surrounding spaces with figure below). (GABA, 250x)

Figure 10.22: Above section fluoresced to demonstrate DAPI stained cell nuclei. Note that the pale blue cell nucleus marked "1" corresponds to the figure above. In addition, the many motoneurons illuminated with DAPI show that interneurons comprise a relatively small portion of hypoglossal neurons. See text for a discussion of results. (DAPI, 250x)

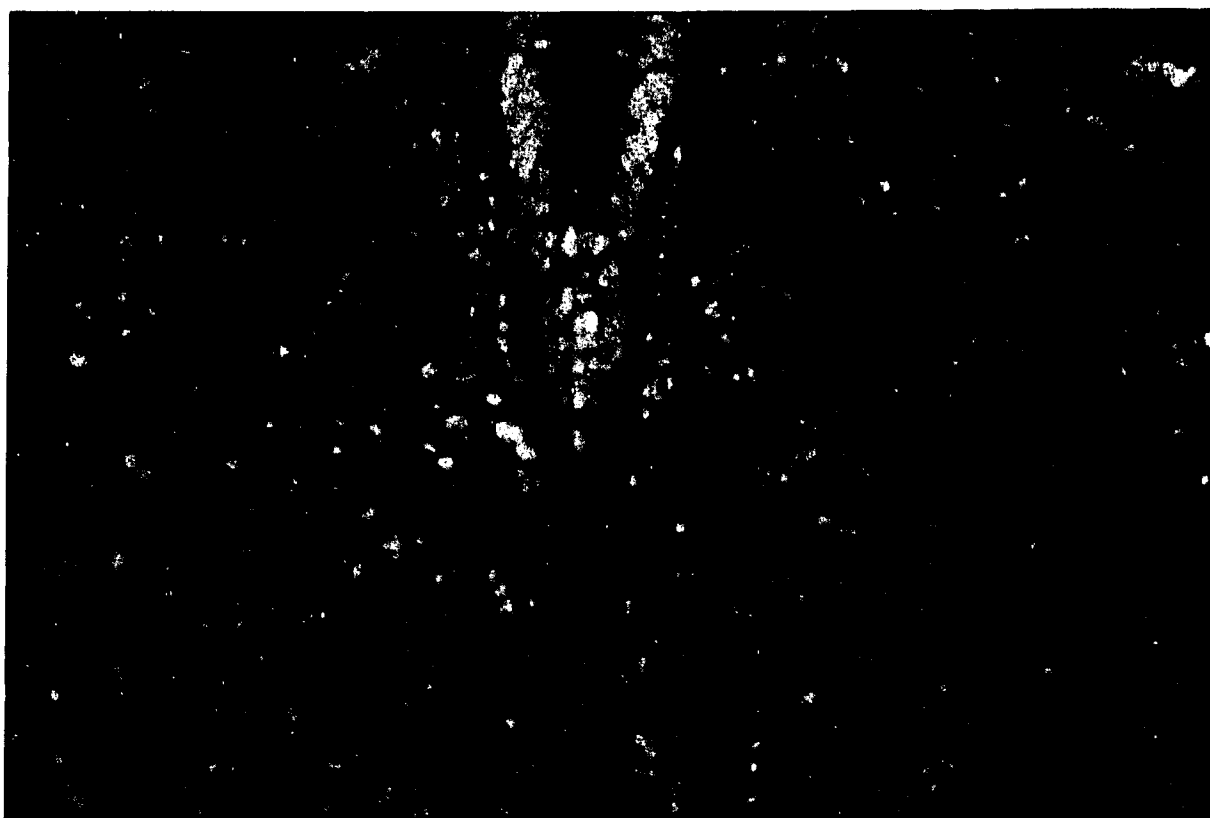
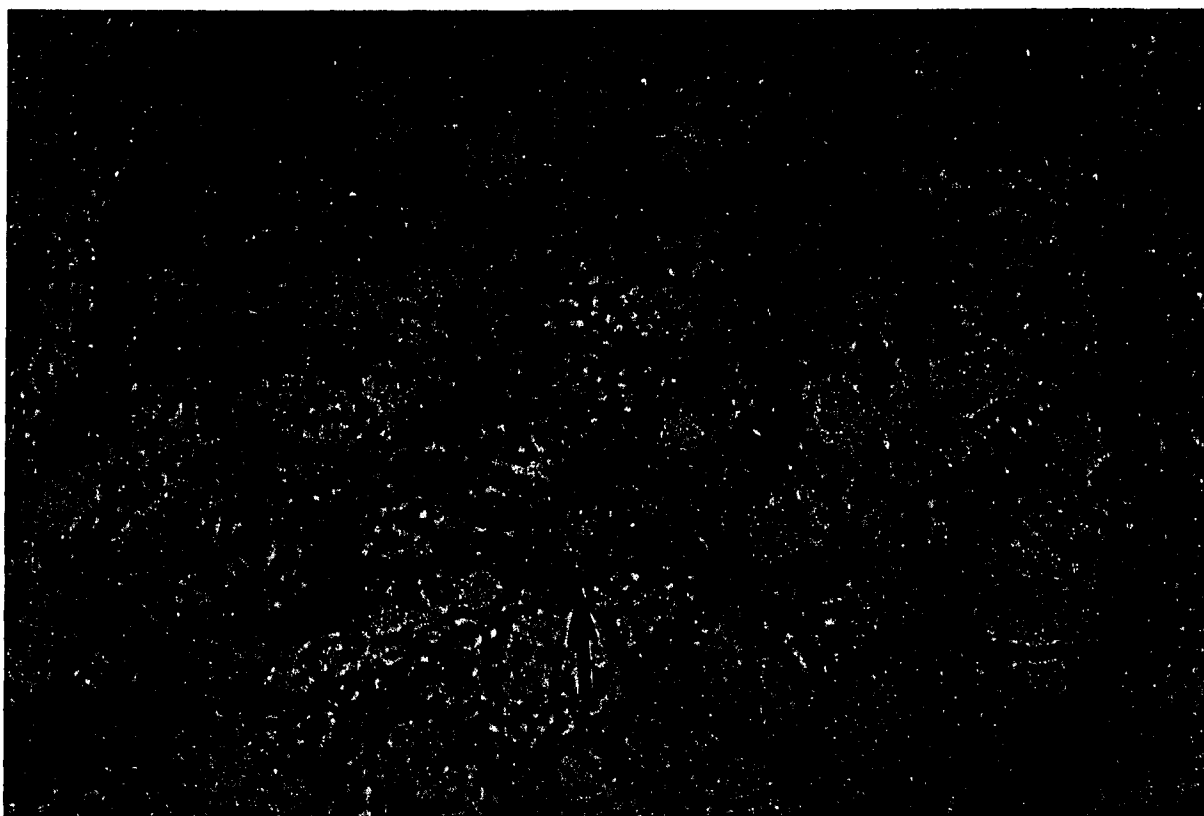


Figure 10.23: Close view of E20 XII stained for GABA. This figure shows that the fiber pattern around the motoneurons (see previous page of figures for reference) is defined by synapses on the motoneurons. These synapses appear as small dots around unstained spaces and are seen throughout the nucleus at this stage (e.g., arrow). This innervation of XII motoneurons is established during the period of cell death (see text). (GABA, 500x)



Chapter 11

Conclusions and Implications for Development and Evolution of the Human URT

11.1 Introduction

The studies in this thesis have sought to define and characterize the ontogenetic development of the nucleus ambiguus and the hypoglossal nucleus. Specifically, this study has described the occurrence of motoneuron cell death in brainstem motor nuclei subserving upper respiratory tract function. Further, this study has demonstrated patterns of neurogenesis, cell migration, nucleus formation, cellular differentiation and afferent innervation in NA and XII. Analyses of these data, and implications for understanding the specific growth and development of NA and XII, have been given in previous chapters. This chapter will place these observations in a broader context, assessing implications for understanding the development of normal and pathological URT neuromuscular control; the possible role of developmental plastic events, e.g., MCD, in the evolution of the human URT; and how these features may relate to origins of our unique capacity for articulate speech.

11.2 Implications for Development of URT Neuromuscular Control

Postnatal URT control is dependent upon specific patterns of innervation of URT muscles by motoneurons of NA and XII. In turn, these URT motoneurons must be specifically innervated by neurons of respiratory, swallowing, and vocalizing centers. These synaptic connections between neuronal and muscular components of the neuromuscular system are established, in large part, prenatally during the period of MCD. Thus, this study, having shown the occurrence of MCD in URT motor nuclei, has defined periods critical to the formation of URT control.

The course of MCD is determined by epigenetic signals from target muscles, afferent neurons and environmental factors. As such, normal patterns of MCD are subject to modification by environmental signals arising within, or external to, the developing organism. Thus, insult to the embryo or fetus during the period of MCD may adversely

affect future neuromuscular control. In particular, insult to the organism during the period of MCD in NA and XII may compromise postnatal URT neuromuscular control. As this study has demonstrated a temporospatial gradient (i.e., differing patterns related to time and brainstem location) in the onset and termination of MCD, developmental "windows" have been established during which NA and/or XII may be preferentially affected by environmental insult. Such preferential insult may create "mismatches" in the patterns of neuromuscular control between peripheral structures and underlie some of the incoordination pathologies seen in the newborn URT.

Prenatal insult capable of altering the course of MCD would include any factor adversely influencing the trophic interactions between developing motoneurons and their target tissues. For example, the introduction of neuromuscular blocking agents to the amniotic sac has been shown to reduce dramatically the amount of cell death (see chapter 3), and may thus result in the persistence of motoneurons that have established improper peripheral connections. While it is highly unlikely that a developing human would be exposed to agents used in these experiments (e.g., curare or α -bungarotoxin), it is likely that individuals may be exposed prenatally to similar agents such as nicotine. Studies have shown that exposure to cigarette smoke or nicotine during pregnancy has adverse effects on the developing fetus (Haglund and Cnattingius, 1990; Bulterys, 1990; Bulterys et.al., 1990). Neonates of mothers exposed to smoke show lower birth weights, greater incidence of neurological handicaps, and an increased rate of perinatal morbidity and mortality (Rantakallio and Koiranen, 1987; Leichter, 1989; Tachi and Aoyama, 1989). Further, specific developmental studies have shown that cigarettes or nicotine reduce fetal breathing movements (Manning and Feyerabend, 1976; Manning et.al., 1978) and damage developing neurons and neural centers (Krous et.al., 1981; Kleshcheva, 1990). Abnormal feedback from affected regions to URT brainstem motor nuclei may thus alter patterns of normal MCD and lead to URT control deficits in the newborn.

One such developmental clinicopathology, associated with prenatal exposure to cigarette smoke, and possibly resulting from abnormal URT control, is the Sudden Infant Death Syndrome (SIDS). SIDS, also known as crib death, is one of the leading causes of death in infants in the United States with a peak incidence between two and four months of age (Hoffman et.al., 1988; Willinger, 1989). It remains essentially a diagnosis of exclusion with both the precise definition of the syndrome (Beckwith, 1970), as well as its specific etiology, still unclear (Bergman et.al., 1970; Guntheroth, 1989). However, developmentally based neuromuscular incoordination in the early postnatal URT has come under increasingly closer scrutiny as to its possible connection to SIDS (Harding, 1984; Sasaki, 1979; Haraguchi et.al., 1983; Laitman, 1986; Friedland et.al., 1992).

In the mid-1970's study of the brains of SIDS victims first revealed neuropathologies in central respiratory control centers of the medulla (proliferation of astroglial fibers in lateral reticular formation) (Naeye, 1976; Naeye et.al., 1989). Subsequent studies have identified other aspects of brainstem pathologies such as the presence of increased dendritic spines in medullary respiratory centers (i.e., dorsal vagal nucleus, nucleus tractus solitarius, reticular formation) (Kinney and Filiano, 1988; Takashima and Becker, 1986). These investigations strongly indicate pathologic insult to developing regions of the brainstem in SIDS victims (Becker, 1983; Becker, 1990). When considered in conjunction with findings of URT respiratory incoordination in apneas, these studies strongly suggest the existence of brainstem pathologies directly affecting URT motor control. Indeed, recent studies (Naeye et.al., 1989; O'Kusky and Norman, 1992) have shown differences within the motoneuron population of the hypoglossal nucleus (XII) of SIDS victims as compared to age-matched controls. The presence in SIDS victims of abnormalities in brainstem motor nuclei subserving URT function may result from insult to the developing motor pool during critical periods of MCD. Such insult would alter the course of MCD, allowing mismatches between URT motoneurons and neuronal and

muscular targets to persist, and thus result in incoordination of URT function in the postnatal organism.

That MCD and SIDS may be related is indicated by the co-temporaneous occurrence of changes in URT neuromuscular patterns and the peak incidence of the syndrome. That is, the peak incidence of SIDS is at approximately three months of age, a period which corresponds to changes in infant breathing habits. At this time, infants, who are primarily nose breathers (largely due to their intra-narial larynx), begin to "experiment" with oral respiration (Sasaki et.al., 1977). The novel neuromuscular activities necessary for this change in breathing patterns may be beyond the capacity of URT motor nuclei developmentally challenged by an abnormal course of MCD. As such, the infant may succumb to respiratory insufficiency due to incoordination in the neuromuscular system subserving oral respiration.

Abnormal MCD may also be manifest at postnatal stages by changes in patterns of innervation. At birth, muscle fibers are innervated by many axons (polyneuronal innervation) which are removed during a period of synapse elimination to establish the mature condition of mononeuronal innervation (Redfern, 1970; Bennett and Pettigrew, 1974; Jansen and Fladby, 1990). Synapse elimination (SE), like MCD, is responsive to muscle activity and serves to refine motor units for proper peripheral control (see review Grinnell and Herrera, 1981). The changes in respiration in the three month human may represent a critical period in the establishment of URT control by defining mature neuromuscular patterns and directing the course of SE. If the motoneurons to the URT have established improper connections during MCD they may adversely influence the course of SE. Further, SE also occurs at neuron-to-neuron synapses and, similarly, may be improperly directed in defining innervation patterns between respiratory and swallowing neurons, and motoneurons of NA and XII. This, in turn, may compound the inability of the newborn to direct URT activity in a coordinated and efficient manner causing functional collapse in the region.

The susceptibility of MCD to epigenetic modification may enable prenatal insults to affect peripheral neuromuscular deficits in the postnatal URT. However, by the same mechanisms of epigenetic modification, developmental changes in central and peripheral centers may refine the course of MCD and evoke novel and highly efficient neuromuscular patterns. The ability for such refinement may have been a necessary prerequisite for the emergence of specialized URT morphologies and activities. In particular, the evolution of the unique, adult human URT may have been dependent upon a plastic neuromuscular system capable of adapting to peripheral change. The following sections will discuss the possible role of MCD in the origins of the human URT and the uniquely human capacity for articulate speech.

11.3 Evolutionary Significance of MCD

11.3.1 General Introduction

Chapter 3 addressed the question of the role of a developmentally "plastic" event such as MCD (i.e., susceptible to epigenetic modification) within the context of a normal developmental milieu. The discussion largely focused on the establishment of specificity and accuracy in peripheral projections thus ensuring coordinated postnatal neuromuscular control. While in individual ontogeny MCD may indeed ensure specificity in the neuromuscular system, it may have a larger role: a mechanism promoting the perpetuation and evolution of species. That is, plastic events such as MCD may promote diversity by allowing for, and subsequently incorporating, genetic variation in complex systems. In particular, the occurrence of MCD in NA and XII may provide a mechanism for the evolution of the human URT and the origin of articulate speech.

11.3.2 Ontogeny and Phylogeny of the Human URT

The adult human URT is unique among mammals in having a larynx positioned low in the neck. This morphology has created an enlarged oro-pharyngeal space which is used for the modification of laryngeal sounds and the production of articulate speech. Interestingly, newborn humans have a larynx positioned high in the neck and thus are incapable of producing the wide array of sounds found in adults. The newborn brain is also immature at birth and even with an adult-like URT, newborns would still be unable to produce fully articulate speech (Lieberman, 1984). The occurrence of MCD in URT motor nuclei may provide insight into similar coordinated developmental sequences between central and peripheral structures.

MCD occurs during a period in which both central neuronal and peripheral muscular systems are not fully developed. That is, MCD begins just after innervation of the target muscle at a time when peripheral muscles are largely immature and have not yet differentiated into distinct fiber types (Rubinstein and Kelley, 1981). However, by the end of MCD, the motor nucleus is well-established and peripheral muscles largely differentiated. Thus, neuronal and muscular components of the neuromuscular system appear to mature co-temporaneously. Further, the course of this development is responsive to epigenetic signals from the innervated, or innervating, tissue (i.e., MCD). Thus, a coordinated developmental sequence has been established by which distinct entities mature together so as to define a single, functional system. An analogous process may be occurring in the newborn human URT. That is, a coordinated developmental sequence may be acting in which functional and morphological changes in the infant URT influence the maturation of brain and brainstem control centers. This would create a URT neuromuscular system able to continue to elicit the neuromuscular patterns of respiration and deglutition, while developing the ability to generate the muscular patterns of articulate speech.

While MCD, and similar plastic events, may function ontogenetically, phylogenetic change in the periphery may also have been "allowed" by reciprocal modification of the neural control centers (Friedland, 1992b). For example, reconstruction of laryngeal position in our early fossil ancestors, the australopithecines, have indicated a URT morphology similar to apes, and unlike that of modern adult humans (Laitman et.al, 1982). Thus, at some time in human evolution, the larynx descended in the neck, and with it came the physical ability to produce articulate speech. However, as in the ontogeny of speech, the brain must also have evolved to be able to generate the neuromuscular patterns necessary for human speech. There are two possibilities for the evolution of a URT-brain system which would have allowed for fully articulate speech: 1) neuronal and muscular changes evolved independent of one another; or 2) neuronal and muscular changes evolved in a dependent, coordinated manner.

Neuronal and peripheral changes in the human speech apparatus probably occurred during similar time periods based upon changes in hominid brain size, inferences from endocasts, comparative neuroanatomical studies and URT reconstruction (Tobias, 1971; Armstrong and Falk, 1982; Laitman et.al., 1992; Lieberman et.al., 1992; Wind et.al., 1992). This would argue for a coordinated evolutionary sequence in speech evolution. Further, the complex neuronal interactions involved in the production of speech would also indicate a coordinated, directed evolution of the neural and peripheral speech apparatus. Thus, it is likely that changes in the morphology of the URT fed back to the brainstem to ensure proper control by the URT motor nuclei. In addition, changes within the nervous system, in higher neuronal centers, may have likewise modified the development of brainstem motor nuclei to maintain peripheral function.

The means by which MCD, and similar events, may have directed and permitted phylogenetic change in the URT, is through individual ontogenies. That is, the occurrence of MCD during prenatal development provides a mechanism by which anatomical changes in the brainstem and periphery, as dictated by the underlying

genome, may ensure a functional system within the individual. For example, as the ancestral human brain increased in size, novel neuronal pathways were developed. For purposes of illustration, suppose a novel pathway from motor cortex to brainstem motor nucleus formed in an individual. If the cortical projection could be integrated into the control mechanism of the URT, the organism may have a competitive advantage over other individuals (obviously this would depend upon the nature of the environment and of the changes in peripheral control). MCD, as it is responsive to epigenetic signals and is thus developmentally plastic, would provide a likely mechanism by which an emerging cortical pathway could establish functional synaptic contact with URT motoneurons and elicit novel neuromuscular patterns in the peripheral URT.

In the same manner, evolving morphological and functional changes in the peripheral URT may have directed the development of NA and XII. For example, as the larynx descended and pathways for respiration and deglutition began to cross in the neck, the organism became more dependent on the neuromuscular system to direct foods toward the esophagus and maintain a patent airway. URT movements which occur prenatally (Humphrey, 1968; Wolfson and Laitman, 1990; Isaacson and Birnholz, 1991) may mimic adult neuromuscular patterns and feed-back to the URT motor nuclei and direct MCD to ensure proper establishment of postnatal control of respiration, deglutition and emerging patterns of vocalization.

One of the most distinctive features of modern humans is their ability to produce articulate speech. The origins of this ability are rooted in changes in URT morphology and in neural control centers. The possibility that these regions evolved independently to form such a complex and specialized system is unlikely, and a coordinated evolutionary sequence is proposed. This sequence would have involved developmental plastic events, such as MCD, which would allow new peripheral and central morphologies to emerge and be integrated into a functional neuromuscular system. The position of motoneurons of NA and XII, between higher neuronal centers and URT muscles establishes these

nuclei as a "link" between evolving systems. Thus, MCD in these nuclei may have served to permit the emergence of central neuronal and peripheral muscular components required for articulate speech.

Appendix A

This section contains eight tables, A.1 to A.8. These tables present the nucleolar counts for the preliminary investigations into MCD. The first four tables show the number of nucleoli counted in each section through NA in two E17 and E19 brainstems. The last four tables present similar data for XII. In addition to presenting data for direct counts, all the following tables show the extrapolation of cell counts at lower sampling rates. These data were shown graphically in figures 5.2 and 5.3 and were used to derive sampling rates for the study of brainstem MCD.

Table A.1
Preliminary Study on Nucleus Ambiguus: E17-A1
Extrapolation of Total Cells at Decreased Sampling Rates

Sampling Rate:	Original Data		Extrapolated Data					
	Every 3rd		Every 6th		Every 9th		Every 12th	
Section #	L	R	L	R	L	R	L	R
0	17	18	17	18	17	18	17	18
3	17	20						
6	18	15	18	15				
9	17	21			17	21		
12	22	28	22	28			22	28
15	23	25						
18	18	48	18	48	18	48		
21	41	38						
24	30	35	30	35			30	35
27	29	33			29	33		
30	33	25	33	25				
33	23	32						
36	21	30	21	30	21	30	21	30
39	28	26						
42	25	23	25	23				
45	17	34			17	34		
48	45	35	45	35			45	35
51	31	22						
54	17	28	17	28	17	28		
57	24	24						
60	21	21	21	21			21	21
63	23	19			23	19		
66	22	30	22	30				
69	22	29						
72	33	17	33	17	33	17	33	17
75	26	23						
78	32	33	32	33				
81	32	24			32	24		
84	35	30	35	30			35	30
87	30	24						
90	27	26	27	26	27	26		
93	24	23						
96	28	18	28	18			28	18
99	19	26			19	26		
102	26	26	26	26				
105	22	21						
108	21	20	21	20	21	20	21	20
111	19	15						
114	20	23	20	23				
117	13	18			13	18		
120	25	10	25	10			25	10
123	9	10						
126	7		7		7			
Nucleolar Counts	1032	1046	543	539	311	362	298	262
Estimated Total Cells	3096	3138	3258	3234	2799	3258	3576	3144

Table A.2
Preliminary Study on Nucleus Ambiguus: E17-A2
Extrapolation of Total Cells at Decreased Sampling Rates

Sampling Rate:	Original Data		Extrapolated Data					
	Every 3rd		Every 6th		Every 9th		Every 12th	
Section #	L	R	L	R	L	R	L	R
0	27	18	27	18	27	18	27	18
3	24	21						
6	29	30	29	30				
9	33	26			33	26		
12	26	24	26	24			26	24
15	18	28						
18	24	26	24	26	24	26		
21	19	27						
24	25	32	25	32			25	32
27	18	20			18	20		
30	15	33	15	33				
33	28	21						
36	19	16	19	16	19	16	19	16
39	22	21						
42	16	36	16	36				
45	19	15			19	15		
48	18	26	18	26			18	26
51	15	25						
54	33	12	33	12	33	12		
57	22	13						
60	19	27	19	27			19	27
63	22	38			22	38		
66	19	26	19	26				
69	33	31						
72	23	27	23	27	23	27	23	27
75	42	14						
78	44	19	44	19				
81	28	18			28	18		
84	24	28	24	28			24	28
87	23	29						
90	20	23	20	23	20	23		
93	29	22						
96	21	26	21	26			21	26
99	22	32			22	32		
102	11	20	11	20				
105	15	21						
108	18	17	18	17	18	17	18	17
111	10	15						
114	14	17	14	17				
117	12	15			12	15		
120	20	6	20	6			20	6
123	10	7						
126	13		13		13			
129	10							
Nucleolar Counts	952	948	478	489	331	303	240	247
Estimated Total Cells	2856	2844	2868	2934	2979	2727	2880	2964

Table A.3
Preliminary Study on Nucleus Ambiguus: E19-A3
Extrapolation of Total Cells at Decreased Sampling Rates

Sampling Rate:	Original Data		Extrapolated Data					
	Every 3rd		Every 6th		Every 9th		Every 12th	
Section #	L	R	L	R	L	R	L	R
0	22	19	22	19	22	19	22	19
3	20	21						
6	17	24	17	24				
9	19	23			19	23		
12	27	16	27	16			27	16
15	16	18						
18	11	18	11	18	11	18		
21	7	17						
24	14	8	14	8			14	8
27	0	11			0	11		
30	14	12	14	12				
33	13	13						
36	22	12	22	12	22	12	22	12
39	9	10						
42	4	13	4	13				
45	11	11			11	11		
48	17	7	17	7			17	7
51	15	18						
54	12	13	12	13	12	13		
57	12	9						
60	11	10	11	10			11	10
63	17	5			17	5		
66	13	15	13	15				
69	13	16						
72	16	11	16	11	16	11	16	11
75	11	19						
78	14	13	14	13				
81	16	8			16	8		
84	17	19	17	19			17	19
87	17	23						
90	18	25	18	25	18	25		
93	29	10						
96	25	14	25	14			25	14
99	24	16			24	16		
102	20	17	20	17				
105	21	28						
108	19	13	19	13	19	13	19	13
111	21	18						
114	20	19	20	19				
117	8	21			8	21		
120	6	0	6	0			6	0
123		6						
Nucleolar Counts	638	619	339	298	215	206	196	129
Estimated Total Cells	1914	1857	2034	1788	1935	1854	2352	1548

Table A.4
Preliminary Study on Nucleus Ambiguus: E19-B1
Extrapolation of Total Cells at Decreased Sampling Rates

Sampling Rate:	Original Data		Extrapolated Data					
	Every 3rd		Every 6th		Every 9th		Every 12th	
Section #	L	R	L	R	L	R	L	R
0	17	9	17	9	17	9	17	9
3	17	16						
6	12	7	12	7				
9	18	22			18	22		
12	11	10	11	10			11	10
15	20	17						
18	18	24	18	24	18	24		
21	17	15						
24	11	19	11	19			11	19
27	16	24			16	24		
30	17	8	17	8				
33	22	23						
36	12	19	12	19	12	19	12	19
39	19	19						
42	14	10	14	10				
45	15	16			15	16		
48	9	18	9	18			9	18
51	18	22						
54	13	14	13	14	13	14		
57	11	23						
60	5	17	5	17			5	17
63	7	11			7	11		
66	12	11	12	11				
69	17	7						
72	24	15	24	15	24	15	24	15
75	18	10						
78	13	11	13	11				
81	15	15			15	15		
84	17	27	17	27			17	27
87	29	16						
90	26	13	26	13	26	13		
93	20	18						
96	13	13	13	13			13	13
99	19	16			19	16		
102	18	15	18	15				
105	18	8						
108	12	10	12	10	12	10	12	10
111	16	10						
114	23	9	23	9				
117	11	12			11	12		
120	7	13	7	13			7	13
123	4	8						
126	11	12	11	12	11	12		
129	9	11						
132	4	5	4	5			4	5
135	4	6			4	6		
Nucleolar Counts	679	654	319	309	238	238	142	175
Estimated Total Cells	2037	1962	1914	1854	2142	2142	1704	2100

Table A.5
Preliminary Study on Hypoglossal Nucleus: E17-A1
Extrapolation of Total Cells at Decreased Sampling Rates

Sampling Rate:	Original Data		Extrapolated Data					
	Every 3rd		Every 6th		Every 9th		Every 12th	
Section #	L	R	L	R	L	R	L	R
0	31	24	31	24	31	24	31	24
3	39	33						
6	34	39	34	39				
9	35	42			35	42		
12	41	33	41	33			41	33
15	41	37						
18	43	44	43	44	43	44		
21	46	43						
24	44	48	44	48			44	48
27	53	47			53	47		
30	51	52	51	52				
33	54	52						
36	55	45	55	45	55	45	55	45
39	35	44						
42	46	40	46	40				
45	37	42			37	42		
48	37	39	37	39			37	39
51	44	46						
54	42	38	42	38	42	38		
57	42	35						
60	47	36	47	36			47	36
63	38	50			38	50		
66	41	46	41	46				
69	44	50						
72	54	39	54	39	54	39	54	39
75	40	49						
78	47	44	47	44				
81	38	28			38	28		
84	42	33	42	33			42	33
87	30	41						
90	32	30	32	30	32	30		
93	23	27						
96	29	31	29	31			29	31
99	37	33			37	33		
102	36	30	36	30				
105	37	28						
108	38	22	38	22	38	22	38	22
111	29	24						
114	20	25	20	25				
117	19	16			19	16		
120	7	13	7	13			7	13
123	6	10						
Nucleolar Counts	1584	1528	817	751	552	500	425	363
Estimated Total Cells	4752	4584	4902	4506	4968	4500	5100	4356

Table A.6
Preliminary Study on Hypoglossal Nucleus: E17-A2
Extrapolation of Total Cells at Decreased Sampling Rates

Sampling Rate:	Original Data		Extrapolated Data					
	Every 3rd		Every 6th		Every 9th		Every 12th	
Section #	L	R	L	R	L	R	L	R
0	22	15	22	15	22	15	22	15
3	24	16						
6	21	23	21	23				
9	51	46			51	46		
12	40	54	40	54			40	54
15	42	34						
18	51	54	51	54	51	54		
21	49	61						
24	32	51	32	51			32	51
27	31	48			31	48		
30	53	43	53	43				
33	49	46						
36	41	39	41	39	41	39	41	39
39	37	42						
42	45	35	45	35				
45	40	40			40	40		
48	50	38	50	38			50	38
51	44	48						
54	35	51	35	51	35	51		
57	42	37						
60	42	45	42	45			42	45
63	39	43			39	43		
66	45	45	45	45				
69	45	37						
72	35	30	35	30	35	30	35	30
75	54	30						
78	42	34	42	34				
81	35	43			35	43		
84	33	31	33	31			33	31
87	41	30						
90	30	20	30	20	30	20		
93	21	33						
96	38	35	38	35			38	35
99	30	29			30	29		
102	25	25	25	25				
105	27	15						
108	16	10	16	10	16	10	16	10
111	13	12						
114	10	9	10	9				
117	12	6			12	6		
120	15	6	15	6			15	6
123	10							
126	3		3		3			
129	5							
Nucleolar Counts	1465	1389	724	693	471	474	364	354
Estimated Total Cells	4395	4167	4344	4158	4239	4266	4368	4248

Table A.7
Preliminary Study on Hypoglossal Nucleus: E19-A3
Extrapolation of Total Cells at Decreased Sampling Rates

Sampling Rate:	Original Data		Extrapolated Data					
	Every 3rd		Every 6th		Every 9th		Every 12th	
Section #	L	R	L	R	L	R	L	R
0	15	11	15	11	15	11	15	11
3	16	11						
6	14	12	14	12				
9	32	15			32	15		
12	31	29	31	29			31	29
15	29	35						
18	28	33	28	33	28	33		
21	35	40						
24	35	36	35	36			35	36
27	44	41			44	41		
30	35	43	35	43				
33	33	39						
36	37	43	37	43	37	43	37	43
39	33	32						
42	40	33	40	33				
45	39	24			39	24		
48	40	32	40	32			40	32
51	29	36						
54	31	36	31	36	31	36		
57	30	39						
60	43	32	43	32			43	32
63	25	29			25	29		
66	32	24	32	24				
69	24	37						
72	36	34	36	34	36	34	36	34
75	37	39						
78	33	34	33	34				
81	34	29			34	29		
84	36	31	36	31			36	31
87	38	26						
90	27	21	27	21	27	21		
93	18	26						
96	22	16	22	16			22	16
99	17	30			17	30		
102	26	22	26	22				
105	19	21						
108	27	25	27	25	27	25	27	25
111	22	21						
114	27	28	27	28				
117	22	20			22	20		
120	24	13	24	13			24	13
123	17	18						
126	15	16	15	16	15	16		
129	9	12						
132		4		4				4
Nucleolar Counts	1256	1228	654	608	429	407	346	306
Estimated Total Cells	3768	3684	3924	3648	3861	3663	4152	3672

Table A.8
Preliminary Study on Hypoglossal Nucleus: E19-B1
Extrapolation of Total Cells at Decreased Sampling Rates

Sampling Rate:	Original Data		Extrapolated Data					
	Every 3rd		Every 6th		Every 9th		Every 12th	
Section #	L	R	L	R	L	R	L	R
0	10	9	10	9	10	9	10	9
3	24	9						
6	19	18	19	18				
9	14	20			14	20		
12	18	25	18	25			18	25
15	27	23						
18	32	33	32	33	32	33		
21	30	47						
24	28	27	28	27			28	27
27	25	32			25	32		
30	31	41	31	41				
33	29	35						
36	26	35	26	35	26	35	26	35
39	29	29						
42	40	34	40	34				
45	31	32			31	32		
48	35	27	35	27			35	27
51	33	37						
54	35	31	35	31	35	31		
57	31	29						
60	49	31	49	31			49	31
63	45	26			45	26		
66	40	31	40	31				
69	26	32						
72	36	39	36	39	36	39	36	39
75	30	37						
78	29	18	29	18				
81	43	35			43	35		
84	39	20	39	20			39	20
87	35	29						
90	30	36	30	36	30	36		
93	38	34						
96	32	26	32	26			32	26
99	30	29			30	29		
102	24	17	24	17				
105	27	19						
108	20	26	20	26	20	26	20	26
111	28	27						
114	19	25	19	25				
117	24	16			24	16		
120	13	11	13	11			13	11
123	5	9						
126	11	7	11	7	11	7		
129	11	8						
132	4	5	4	5			4	5
135	6	4			6	4		
Nucleolar Counts	1241	1170	620	572	418	410	310	281
Estimated Total Cells	3723	3510	3720	3432	3762	3690	3720	3372

Appendix B

This section contains 25 tables, B.1 to B.25. These tables present the nucleolar counts for all specimens used in the investigation of MCD in NA. All ages, except adults, are shown by litter. The following counts were multiplied by the sampling rate to generate estimates of motoneuron numbers in NA as discussed in chapter 6.

Table B.1
Nucleus Ambiguus: E17, Litter "A"

Nucleolar Counts Per Section

Section Number	E17-A3		E17-A4		E17-A5		E17-A6	
	Left	Right	Left	Right	Left	Right	Left	Right
1	23	21	21	23	22	25	15	13
2	24	37	23	20	22	13	26	22
3	27	34	20	17	21	24	16	25
4	24	20	31	35	26	15	16	16
5	25	16	17	21	24	18	18	19
6	20	21	27	28	23	22	8	9
7	17	26	21	25	19	29	19	27
8	18	14	17	29	27	6	14	16
9	18	14	19	16	17	24	13	23
10	11	8	15	15	22	12	23	12
11	10	18	17	14	17	28	14	10
12	15	29	14	14	18	19	10	15
13	21	16	10	21	15	13	18	17
14	24	15	28	18	13	15	6	6
15	25	25	22	20	14	24	13	22
16	17	15	14	17	20	8	13	7
17	22	18	15	15	21	20	15	26
18	26	16	18	25	12	9	14	11
19	12	15	18	24	14	11	17	14
20	5	10	19	22	19	17	16	22
21	7	11	17	24	21	17	17	24
22	13	13	17	26	9	24	26	17
23	12	10	15	17	18	22	20	22
24	11	9	17	8	24	20	16	12
25	6	7	18	15	14	10	13	28
26	19	11	19	17	15	19	10	15
27	13	23	20	19	17	18	14	25
28	6	13	9	10	23	23	11	12
29	16	18	16	13	31	21	10	9
30	11	10	10	7	18	18	12	16
31	10	9	11	11	17	16	14	14
32	8	12	6	9	10	7	9	7
33	5				13	10	12	12
34	4				15	15	10	18
35					8	8	14	15
36					6	3	20	16
37					3	5	11	14
38					9	6	9	12
39					3	5	5	6
40					2		6	7
41							2	10
								3

Table B.2
Nucleus Ambiguus: E17, Litter "B"

Nucleolar Counts Per Section

Section Number	E17-B5		E17-B6	
	Left	Right	Left	Right
1	17	15	10	25
2	37	23	24	26
3	21	18	22	19
4	42	22	36	20
5	30	20	18	15
6	25	19	25	15
7	18	18	18	13
8	16	15	12	28
9	21	18	14	23
10	11	14	10	16
11	20	9	24	23
12	14	18	7	18
13	10	14	8	16
14	12	18	11	11
15	11	12	11	18
16	5	21	22	22
17	9	20	6	18
18	31	24	13	14
19	17	15	19	19
20	18	9	20	17
21	22	23	11	12
22	14	20	13	8
23	10	13	12	12
24	14	12	7	10
25	22	13	11	6
26	17	25	6	4
27	10	16	8	4
28	7	5	7	4
29	15	10	5	3
30	11	6	6	8
31	9	3	7	3
32	10	9	4	5
33	8	10	6	7
34	5	8	5	8
35	8	6	3	3
36	8	6	7	5
37	4		4	2
38	5			

Table B.3
Nucleus Ambiguus: E17, Litter "C"

Nucleolar Counts Per Section

Section Number	E17-C2		E17-C3		E17-C4		E17-C5	
	Left	Right	Left	Right	Left	Right	Left	Right
1	16	14	14	16	14	13	16	14
2	21	21	22	22	16	19	19	26
3	18	22	25	27	16	23	18	24
4	25	23	25	26	18	18	23	32
5	26	22	18	23	18	18	30	18
6	25	24	25	23	23	18	24	20
7	13	12	11	11	14	15	26	21
8	11	18	13	21	15	13	14	18
9	16	17	15	15	26	13	17	17
10	21	11	13	9	17	20	19	18
11	24	16	19	16	11	18	13	18
12	14	16	14	12	10	10	16	11
13	22	19	13	15	10	23	16	14
14	15	25	14	19	16	11	14	8
15	13	18	15	13	10	15	15	17
16	14	20	14	18	19	13	27	13
17	17	17	17	14	14	14	11	16
18	22	27	20	30	19	10	23	21
19	28	21	26	28	23	6	25	24
20	24	22	23	29	13	10	14	16
21	17	18	18	19	9	27	26	18
22	18	13	12	12	12	13	10	20
23	15	14	9	10	14	10	10	14
24	18	16	12	17	9	11	19	21
25	12	14	14	3	5	5	17	7
26	11	10	12	4	11	9	7	14
27	12	12	12	9	9	10	13	12
28	16	13	15	4	10	14	13	12
29	7	9	3	6	15	7	12	16
30	13	11	9	10	5	10	14	21
31	10	5	7	10	17	11	15	15
32	11	8	9		12	8	14	12
33	6	6	7		19	4	9	7
34	5	9	6		16	4	8	10
35	3				8	5	4	3
36					6	5	10	3
37					6	3	6	
38					4		6	
39					4		6	
40					2			
41					3			

Table B.4
Nucleus Ambiguus: E18, Litter "D"

Nucleolar Counts Per Section

Section Number	E18-D2		E18-D4		E18-D5	
	Left	Right	Left	Right	Left	Right
1	12	17	20	19	14	13
2	25	25	15	22	20	10
3	25	22	24	27	19	17
4	23	23	27	27	17	18
5	16	20	18	20	19	23
6	12	15	20	26	17	24
7	17	28	15	15	13	23
8	16	21	23	19	19	11
9	13	17	19	22	14	15
10	11	9	17	15	14	10
11	6	4	18	26	17	14
12	11	12	24	15	15	13
13	3	9	15	13	15	6
14	11	9	17	9	5	6
15	8	13	10	10	11	11
16	12	15	14	11	8	15
17	7	16	15	12	11	14
18	5	12	8	14	11	8
19	8	10	12	8	14	19
20	14	10	16	15	15	15
21	11	13	21	18	13	10
22	10	15	9	7	14	14
23	13	19	8	10	9	17
24	10	7	10	14	16	16
25	6	6	5	10	7	9
26	12	9	12	10	16	13
27	6	16	13	14	6	8
28	8	9	12	9	7	8
29	8	10	10	11	12	11
30	8	9	8	11	10	7
31	8	10	13	7	9	7
32	5	7	6	8	8	5
33	9	6	7	8	14	8
34	5	9	5	15	12	19
35	9	11	5	10	13	14
36	3	9	3	12	10	7
37	4	6		2	13	6
38	3	4			13	5
39		1			7	12
40					4	6
41					3	4
						1

Table B.5
Nucleus Ambiguus: E18, Litter "E"

Nucleolar Counts Per Section

Section Number	E18-E2		E18-E4		E18-E5		E18-E6	
	Left	Right	Left	Right	Left	Right	Left	Right
1	18	16	21	18	26	27	24	16
2	14	23	25	22	28	32	19	18
3	24	21	22	24	20	33	19	27
4	30	20	22	19	31	21	21	25
5	23	24	9	18	18	22	31	20
6	15	17	9	14	22	23	21	17
7	22	14	13	24	22	17	21	15
8	24	15	10	20	18	21	20	24
9	20	15	13	24	19	23	23	22
10	15	10	6	14	13	23	13	17
11	14	7	16	13	15	16	15	25
12	13	11	9	10	9	14	19	16
13	12	8	9	11	7	21	12	12
14	7	4	17	11	15	5	10	20
15	15	12	6	12	10	9	9	16
16	13	19	8	13	17	14	5	21
17	9	10	7	8	12	15	17	16
18	6	15	20	14	13	14	18	14
19	14	17	23	15	15	10	13	9
20	10	21	17	14	16	15	10	23
21	18	11	21	14	27	22	20	13
22	7	6	7	12	17	15	18	16
23	13	20	18	16	10	17	8	18
24	17	10	6	10	20	12	6	14
25	14	8	9	14	18	14	15	8
26	9	17	16	4	10	31	6	12
27	9	10	10	6	8	17	11	11
28	4	9	10	10	9	10	9	13
29	13	7	14	5	11	14	10	10
30	15	9	5	14	10	17	9	19
31	10	15	8	7	21	14	13	12
32	9	7	22	11	7	12	8	18
33	4	11	12	21	15	14	13	15
34	6	8	10	11	16	6	8	15
35	11	12	15	10	10	8	10	15
36	9	7	17	6	10	15	9	17
37	11	5	5	6	8	4	5	10
38	5	10	7	6	4	12	3	7
39	5	6	3	7	4	7	8	10
40	7	6	4	0	6	4	5	2
41	2	4	0	2	7	5	4	7
42	1	5	1	3				0
43	3	7						1
44	1	3						
45		1						
46		2						

Table B.6
Nucleus Ambiguus: E18, Litter "F"

Nucleolar Counts Per Section

Section Number	E18-F3		E18-F4		E18-F5	
	Left	Right	Left	Right	Left	Right
1	10	15	23	24	12	9
2	24	14	19	29	13	16
3	25	13	18	23	16	17
4	23	29	13	19	19	17
5	16	20	24	23	15	20
6	18	14	12	23	17	20
7	11	13	12	19	17	12
8	13	13	14	26	20	21
9	8	11	13	14	15	13
10	16	17	12	15	17	20
11	15	9	10	18	17	14
12	15	21	15	11	9	18
13	9	19	25	12	13	16
14	10	6	17	13	11	9
15	5	11	13	20	4	10
16	5	12	11	14	9	18
17	8	11	20	23	15	25
18	6	9	13	15	12	21
19	5	13	10	19	18	12
20	17	15	16	20	15	12
21	17	10	23	23	12	14
22	8	14	16	14	17	14
23	10	14	20	21	18	21
24	12	11	16	16	10	13
25	6	8	6	12	14	13
26	7	5	18	11	14	11
27	9	14	9	13	15	8
28	9	10	8	15	13	21
29	8	4	15	7	11	16
30	5	7	13	19	10	8
31	4	6	15	12	24	20
32	7	2	13	5	15	18
33	9	7	18	11	13	29
34	6	6	9	4	20	11
35	10	5	4		12	12
36	7	1	3		14	7
37	6	7			9	6
38	5	11				
39	6					

Table B.7
Nucleus Ambiguus: E19, Litter "D"

Nucleolar Counts Per Section

Section Number	E19-D1		E19-D2		E19-D4	
	Left	Right	Left	Right	Left	Right
1	11	9	14	18	15	10
2	16	20	16	15	11	10
3	18	21	15	19	22	15
4	18	16	20	19	18	17
5	19	4	16	12	16	17
6	16	16	16	11	16	14
7	17	12	16	13	15	9
8	14	21	20	10	21	23
9	5	14	8	10	12	13
10	11	16	11	17	8	7
11	16	13	6	12	14	18
12	14	13	6	10	10	6
13	9	5	17	16	11	12
14	16	18	9	5	11	11
15	8	9	8	6	7	23
16	7	8	7	3	7	10
17	9	11	4	7	6	12
18	15	12	13	13	11	10
19	11	4	12	4	2	7
20	18	8	4	8	12	11
21	13	7	3	8	11	19
22	13	13	2	3	11	8
23	11	7	4	8	11	11
24	6	10	4	5	10	9
25	17	5	9	6	7	13
26	6	4	7	7	12	10
27	6	3	8	4	7	8
28	5	6	9	9	13	11
29	8	6	8	4	9	9
30	8	10	14	10	20	11
31	5	5	12	12	5	9
32	9	7	12	13	8	8
33	12	13	5	7	10	14
34	7	7	5	12	11	13
35	10	15	5	4	9	11
36	2	7	6	13	8	12
37	8	4	1	3	9	10
38	4	3		1	8	6
39	7	2			10	4
40	5	2			8	5
41	4				4	5
42					3	3
43					2	1

Table B.8
Nucleus Ambiguus: E19, Litter "E"

Nucleolar Counts Per Section

Section Number	E19-E3		E19-E5		E19-E6	
	Left	Right	Left	Right	Left	Right
1	15	9	19	10	19	7
2	18	14	30	13	13	11
3	14	13	18	15	13	20
4	17	20	18	13	17	14
5	9	17	20	13	10	20
6	16	14	17	8	19	18
7	16	10	15	9	21	14
8	7	10	12	17	19	10
9	10	18	12	12	17	13
10	11	13	14	16	15	9
11	18	15	11	14	16	10
12	8	12	15	7	7	10
13	9	11	12	8	8	10
14	6	10	8	9	10	14
15	10	16	10	8	8	14
16	5	10	10	6	9	12
17	8	6	9	6	9	8
18	14	6	17	11	10	8
19	9	15	6	4	14	4
20	10	15	6	13	11	7
21	18	15	13	11	16	17
22	14	9	10	7	11	10
23	18	14	8	8	7	19
24	12	16	8	5	16	8
25	11	13	8	4	11	13
26	12	5	4	8	8	6
27	6	9	5	8	5	5
28	7	5	8	3	10	5
29	9	9	5	7	9	6
30	6	3	4	7	9	6
31	15	6	6	9	7	12
32	10	17	10	8	10	9
33	7	7	8	10	9	10
34	12	12	16	7	16	12
35	12	5	6	11	8	14
36	8	4	12	7	5	8
37	8	5	11	6	8	7
38	7	7	4	6	8	6
39	6	9	2	7	5	7
40	2	0	3	5	0	6
41	3	2	5	5	4	5
42	5		4	5		
43	1			5		
44	3					

Table B.9
Nucleus Ambiguus: E19, Litter "F"

Nucleolar Counts Per Section

Section Number	E19-F1		E19-F2		E19-F4		E19-F7	
	Left	Right	Left	Right	Left	Right	Left	Right
1	15	16	20	19	11	13	16	7
2	23	14	17	14	13	16	12	16
3	20	14	20	20	18	23	15	12
4	11	13	23	16	17	20	10	17
5	10	14	15	22	12	16	17	15
6	16	14	14	14	7	15	16	6
7	9	16	6	19	16	13	9	20
8	13	10	13	9	13	16	11	8
9	9	10	14	10	14	19	15	6
10	7	9	17	11	10	8	8	7
11	6	7	11	11	6	11	9	10
12	11	8	12	14	11	10	9	13
13	9	5	7	3	13	12	10	9
14	4	6	11	7	7	15	9	9
15	5	10	33	7	10	6	8	6
16	7	14	12	12	7	10	10	13
17	12	11	16	21	7	8	8	11
18	16	14	10	14	12	6	14	9
19	10	8	19	24	8	19	10	13
20	10	14	13	13	8	15	12	13
21	6	10	12	11	11	10	13	14
22	5	8	9	12	10	12	15	15
23	9	5	5	4	4	6	10	19
24	3	7	11	4	5	11	7	12
25	13	8	6	10	9	10	8	9
26	10	12	10	8	8	12	4	4
27	9	14	14	8	10	8	10	17
28	11	9	7	10	8	7	12	11
29	12	11	6	7	9	11	9	13
30	11	11	7	9	10	13	10	11
31	7	3	6	7	11	9	5	10
32	4	5	7	8	16	17	12	14
33	8	6	9	5	10	11	7	14
34	5	4	8	6	9	18	7	11
35	5	3	2	2	13	7	7	7
36	2	3	3	9	11	10	8	4
37	2	1	4	8	7	9	8	12
38		1	2	7	10	3	6	3
39				1	5	5	6	2
40				1	4	7	7	2
41					3	5	3	
42					0	3		
43					2	2		
44					2			

Table B.10
Nucleus Ambiguus: E20, Litter "A"

Nucleolar Counts Per Section

Section Number	E20-A1		E20-A2		E20-A6	
	Left	Right	Left	Right	Left	Right
1	12	25	8	10	13	8
2	22	29	13	12	11	14
3	15	12	12	16	11	13
4	18	15	16	14	15	14
5	15	13	20	13	13	14
6	16	13	14	11	8	12
7	12	14	14	10	15	17
8	10	9	8	14	8	16
9	8	14	11	6	7	13
10	6	13	20	5	13	14
11	8	5	9	14	9	11
12	15	9	17	5	16	5
13	7	7	8	6	6	14
14	9	6	6	7	7	13
15	7	6	5	7	9	8
16	9	8	8	3	10	6
17	6	9	5	6	4	5
18	8	8	9	7	7	8
19	12	8	14	8	5	5
20	19	4	9	11	5	8
21	3	5	6	12	9	8
22	4	9	5	8	10	4
23	10	5	6	12	7	6
24	4	5	5	7	9	11
25	8	9	8	5	10	13
26	8	10	11	4	6	9
27	6	11	8	6	6	14
28	5	8	8	9	5	11
29	6	7	7	9	6	4
30	7	6	10	10	5	5
31	5	11	9	12	8	8
32	4	6	5	8	9	12
33	5	3	6	7	8	5
34	6	3	3	4	6	4
35	3	5	8	2	7	6
36	0	3	2	4	7	7
37	3	2	4	3	6	6
38		2	4	3	4	6
39			0	3	5	8
40			2	3	7	11
41			2	2	3	8
42			1	5	1	3
43				1	2	4
44					4	7
45					2	2
46					0	4
47					3	

Table B.11
Nucleus Ambiguus: E20, Litter "B"

Nucleolar Counts Per Section

Section Number	E20-B2		E20-B3		E20-B4		E20-B5	
	Left	Right	Left	Right	Left	Right	Left	Right
1	13	7	23	10	5	8	5	4
2	7	16	13	16	16	15	7	9
3	9	15	10	19	11	11	17	12
4	12	12	11	13	19	14	15	15
5	13	16	7	12	17	18	13	13
6	11	16	6	14	11	18	14	10
7	12	14	10	17	15	8	11	10
8	12	14	10	11	11	15	11	4
9	8	11	13	12	14	11	12	13
10	12	15	6	5	13	10	14	5
11	7	9	7	6	14	11	12	4
12	9	8	2	11	9	4	5	8
13	10	10	7	3	5	7	7	6
14	9	8	5	8	4	10	5	3
15	10	9	8	10	5	9	7	8
16	5	7	14	4	5	6	3	11
17	7	8	6	7	8	11	10	4
18	14	5	9	5	5	9	5	10
19	6	10	7	11	6	10	2	8
20	3	13	11	12	5	7	4	6
21	9	12	9	8	6	7	6	5
22	8	10	4	6	8	5	5	7
23	13	12	8	3	6	7	3	7
24	8	8	4	6	5	9	8	9
25	9	10	5	7	5	7	8	6
26	9	8	9	8	3	7	9	8
27	10	7	6	6	6	5	4	8
28	12	4	5	7	9	13	9	5
29	13	10	6	6	10	7	6	4
30	6	6	11	12	8	8	9	8
31	6	4	6	9	3	9	5	5
32	14	7	7	8	6	5	8	6
33	6	9	9	7	5	8	8	4
34	8	8	8	8	6	6	3	7
35	3	6	6	5	7	7	5	6
36	7	5	5	9	6	10	8	7
37	7	5	4	4	10	10	5	6
38	8	6	5	4	11	7	12	5
39	4	3	3	2	6	11	9	5
40	4	2	4	1	8	7	3	3
41	7	5		3	7	16	3	3
42	3	7			8	8	3	7
43	5	3			5	8	0	
44					2	6	2	
45					4	5		
46					3	0		
47					2	1		
48					1			

Table B.12
Nucleus Ambiguus: E20, Litter "C"

Nucleolar Counts Per Section

Section Number	E20-C2		E20-C4		E20-C5	
	Left	Right	Left	Right	Left	Right
1	17	12	12	9	9	14
2	12	10	11	8	11	18
3	8	6	15	13	15	12
4	11	7	11	10	10	18
5	7	15	11	15	15	17
6	10	11	10	13	12	8
7	12	12	7	11	10	11
8	7	10	13	8	12	12
9	4	16	7	12	13	17
10	4	9	4	10	7	11
11	7	7	6	9	11	8
12	3	2	4	7	3	11
13	6	6	4	3	4	7
14	6	5	8	5	10	4
15	7	7	5	6	4	7
16	8	6	5	4	3	4
17	8	7	2	12	6	8
18	7	5	4	5	6	9
19	6	8	8	3	7	7
20	2	10	10	8	3	6
21	5	5	8	6	8	9
22	8	6	3	4	6	7
23	7	3	6	6	8	9
24	7	5	3	3	7	9
25	7	5	10	6	10	7
26	8	7	3	7	7	5
27	7	5	4	4	6	4
28	4	5	5	6	5	4
29	4	3	6	6	7	6
30	9	5	5	5	8	6
31	4	3	6	6	6	4
32	5	5	8	4	4	2
33	4	2	9	7	5	4
34	1	2	6	4	12	7
35		4	5	5	8	6
36		2	8	5	6	7
37			4	6	7	7
38			7	5	5	5
39			4	2	4	6
40			3	4	3	4
41				2	5	3
42				0	2	3
43				1		2

Table B.13
Nucleus Ambiguus: P1, Litter "E"

Nucleolar Counts Per Section

Section Number	P1-E2		P1-E5	
	Left	Right	Left	Right
1	16	15	11	9
2	14	16	13	8
3	12	17	15	14
4	8	12	12	11
5	18	10	11	9
6	9	7	14	8
7	7	9	16	9
8	10	7	11	10
9	10	5	4	16
10	7	6	9	14
11	5	6	17	6
12	12	8	5	7
13	10	3	6	10
14	7	6	7	3
15	4	11	2	10
16	4	9	8	13
17	11	9	5	3
18	9	3	8	3
19	5	7	6	4
20	13	8	5	11
21	10	6	10	10
22	6	5	4	8
23	1	6	11	6
24	2	3	9	5
25	2	5	5	4
26	11	1	6	3
27	4	2	9	4
28	4	4	8	5
29	9	5	6	4
30	6	5	11	9
31	3	3	6	1
32	2	9	5	6
33	1	2	4	8
34	1	1	5	4
35		5	2	2
36			1	3
37				1

Table B.14
Nucleus Ambiguus: P1, Litter "F"

Nucleolar Counts Per Section

Section Number	P1-F1		P1-F2		P1-F3		P1-F4	
	Left	Right	Left	Right	Left	Right	Left	Right
1	17	13	18	13	17	10	18	16
2	19	24	19	11	15	12	25	23
3	15	16	26	20	11	11	20	29
4	19	14	20	26	12	11	25	15
5	16	18	16	20	11	10	16	15
6	10	7	11	12	23	12	20	14
7	1	12	14	18	15	18	6	14
8	12	7	5	12	13	14	8	10
9	12	9	15	9	7	11	6	14
10	13	11	15	5	4	12	12	12
11	9	6	7	12	6	10	5	13
12	5	9	7	11	8	4	10	16
13	7	2	4	16	3	11	9	4
14	2	5	12	8	7	13	7	4
15	5	5	8	7	4	8	4	5
16	9	13	13	11	4	7	5	6
17	4	8	13	2	6	10	10	8
18	10	11	8	14	9	5	11	8
19	9	9	6	13	10	14	9	9
20	6	8	13	6	6	12	5	10
21	6	5	9	7	6	6	11	8
22	5	5	12	9	11	10	1	5
23	1	2	6	4	10	7	4	5
24	7	2	5	7	7	8	5	3
25	5	7	6	4	3	5	7	4
26	4	5	6	6	8	5	6	5
27	10	6	10	4	6	5	7	3
28	7	5	5	4	4	9	4	4
29	5	2	3	5	6	7	3	6
30	3	2	5	3	3	12	4	3
31	4	4	5	4	8	6	5	4
32	1	7	3	7	2	4	3	5
33		2	3		3	4		6
34		4						3
35		6						2
36		3						

Table B.15
Nucleus Ambiguus: P1, Litter "G"

Nucleolar Counts Per Section

Section Number	P1-G3		P1-G5		P1-G6		P1-G7	
	Left	Right	Left	Right	Left	Right	Left	Right
1	22	17	20	18	30	13	25	21
2	22	27	22	20	20	17	27	20
3	15	16	15	17	24	13	24	18
4	10	21	14	27	11	10	18	25
5	17	19	15	17	2	10	9	19
6	15	16	7	10	10	10	8	12
7	17	12	18	11	15	11	23	8
8	10	8	12	11	8	8	6	10
9	6	10	11	16	16	8	8	9
10	5	6	15	5	9	7	6	5
11	13	8	6	7	7	9	7	5
12	8	7	4	15	15	17	10	8
13	11	8	5	10	11	8	6	10
14	13	5	21	12	8	5	16	7
15	16	10	12	12	3	10	13	10
16	6	15	8	8	6	7	15	10
17	9	3	11	10	8	3	8	6
18	12	7	3	6	6	5	5	9
19	8	10	7	10	5	2	3	7
20	8	7	4	5	2	4	8	10
21	6	6	6	4	2	2	8	5
22	8	15	3	2		4	9	5
23	5	6	4	4		2	4	7
24	8	5	2	2			2	6
25	2	4	3				0	1
26	2	4					2	
27	2	2						
28	1	3						
29		2						
30		4						

Table B.16
Nucleus Ambiguus: A8

Nucleolar Counts Per Section

Section Number	A8		Section Number	A8	
	Left	Right		Left	Right
1	2	6	56	4	5
2	7	6	57	5	6
3	10	6	58	3	7
4	12	8	59	5	7
5	11	12	60	8	6
6	14	9	61	8	7
7	12	9	62	5	7
8	10	16	63	8	4
9	18	10	64	4	8
10	11	9	65	6	7
11	9	7	66	3	7
12	12	12	67	5	5
13	16	12	68	4	2
14	7	9	69	2	4
15	11	9	70	3	2
16	8	9	71	1	4
17	7	5	72	3	4
18	10	7	73	4	3
19	8	8	74	6	2
20	9	6	75	4	6
21	8	12	76	4	7
22	5	5	77	0	5
23	4	7	78	2	5
24	8	7	79	3	4
25	10	4	80	4	3
26	6	5	81	3	5
27	8	5	82	3	5
28	6	4	83	4	2
29	5	3	84	2	2
30	6	4	85	3	5
31	10	8	86	3	1
32	0	5	87	1	2
33	5	3	88	4	4
34	3	2	89	3	0
35	7	5	90	4	1
36	9	9	91		4
37	2	4			
38	2	2			
39	6	3			
40	3	8			
41	8	9			
42	1	6			
43	2	3			
44	2	6			
45	4	7			
46	6	5			
47	2	6			
48	11	4			
49	5	3			
50	3	8			
51	5	6			
52	4	4			
53	4	11			
54	8	10			
55	3	7			

Table B.17
Nucleus Ambiguus: A9

Nucleolar Counts Per Section

Section Number	A9		Section Number	A9	
	Left	Right		Left	Right
1	9	8	56	6	8
2	9	6	57	5	3
3	10	11	58	7	7
4	9	16	59	4	3
5	12	12	60	12	3
6	13	14	61	5	4
7	13	14	62	7	2
8	12	8	63	4	4
9	10	10	64	10	11
10	15	10	65	4	5
11	13	7	66	2	9
12	13	8	67	11	4
13	7	7	68	6	6
14	7	6	69	5	5
15	11	5	70	8	6
16	8	7	71	4	3
17	8	6	72	7	2
18	5	5	73	6	3
19	9	6	74	5	9
20	7	16	75	6	1
21	11	12	76	4	4
22	13	9	77	7	5
23	12	4	78	4	2
24	6	2	79	3	2
25	5	5	80	2	4
26	4	7	81	7	1
27	1	6	82	4	3
28	2	7	83	1	2
29	5	11	84	2	4
30	5	11	85	3	5
31	9	5	86	5	4
32	12	10	87	6	2
33	8	11	88	8	3
34	13	7	89	2	5
35	8	8	90	6	2
36	5	9	91	2	4
37	3	2	92	3	5
38	2	3	93	3	1
39	3	8	94	4	1
40	1	14	95	2	5
41	2	15	96	2	2
42	5	9	97	3	
43	8	5	98	2	
44	9	6	99	4	
45	4	7			
46	6	9			
47	8	5			
48	6	10			
49	3	7			
50	4	9			
51	5	4			
52	5	6			
53	9	5			
54	5	7			
55	6	9			

Table B.18
Nucleus Ambiguus: A10

Nucleolar Counts Per Section

Section Number	A10		Section Number	A10	
	Left	Right		Left	Right
1	10	13	56	5	4
2	11	13	57	5	2
3	12	9	58	2	5
4	12	11	59	7	2
5	11	17	60	11	2
6	15	19	61	6	2
7	8	12	62	5	6
8	14	9	63	5	5
9	5	10	64	5	7
10	10	11	65	1	4
11	6	10	66	2	2
12	4	7	67	2	9
13	8	3	68	4	5
14	12	7	69	2	4
15	12	3	70	1	2
16	8	1	71	3	1
17	5	2	72	2	5
18	3	2	73	2	3
19	3	6	74	4	3
20	2	6	75	4	6
21	2	3	76	3	4
22	3	4	77	2	3
23	3	8	78	4	2
24	1	7	79	5	2
25	7	4	80	5	2
26	4	2	81	4	4
27	1	5	82	5	5
28	2	5	83	3	1
29	4	2	84	2	3
30	3	3	85	5	3
31	7	2	86	1	3
32	6	4	87	4	4
33	4	5	88	6	2
34	6	4	89	1	4
35	8	4	90	3	
36	3	4	91	5	
37	4	5	92	1	
38	4	3			
39	6	3			
40	6	2			
41	5	4			
42	4	6			
43	4	2			
44	3	5			
45	6	4			
46	0	4			
47	3	4			
48	3	4			
49	6	3			
50	4	5			
51	5	6			
52	5	4			
53	5	3			
54	4	3			
55	5	5			

Table B.19
Nucleus Ambiguus: A11

Nucleolar Counts Per Section

Section Number	A11		Section Number	A11	
	Left	Right		Left	Right
1	13	7	56	5	4
2	13	9	57	5	0
3	14	11	58	6	5
4	9	13	59	3	3
5	16	15	60	4	4
6	11	10	61	6	3
7	9	15	62	4	3
8	12	12	63	3	4
9	10	9	64	4	2
10	12	12	65	3	6
11	10	9	66	5	6
12	7	10	67	3	2
13	6	12	68	3	5
14	8	5	69	5	3
15	2	8	70	4	3
16	4	6	71	4	3
17	6	10	72	2	1
18	8	4	73	1	2
19	5	3	74	3	3
20	6	3	75	1	7
21	4	3	76	3	3
22	2	10	77	3	7
23	1	7	78	2	2
24	1	7	79	2	2
25	2	6	80	4	1
26	2	5	81	4	2
27	3	6	82	1	3
28	5	6	83	0	1
29	4	7	84	3	4
30	4	3	85	1	2
31	5	2	86	1	
32	3	4	87	2	
33	5	2	88	2	
34	4	2			
35	4	2			
36	4	5			
37	5	3			
38	5	2			
39	4	5			
40	4	4			
41	5	3			
42	3	3			
43	5	1			
44	4	2			
45	3	1			
46	5	2			
47	4	4			
48	6	3			
49	3	4			
50	2	4			
51	5	2			
52	4	3			
53	7	6			
54	2	4			
55	4	3			

Table B.20
Nucleus Ambiguus: A12

Nucleolar Counts Per Section

Section Number	A12		Section Number	A12	
	Left	Right		Left	Right
1	6	12	56	5	3
2	10	16	57	7	3
3	11	5	58	8	6
4	18	6	59	4	5
5	13	19	60	2	5
6	13	18	61	2	5
7	14	12	62	3	5
8	10	13	63	2	5
9	8	11	64	6	7
10	11	7	65	9	6
11	9	8	66	10	6
12	8	7	67	4	4
13	3	5	68	8	3
14	4	9	69	2	1
15	8	6	70	5	3
16	6	5	71	8	2
17	7	9	72	3	9
18	5	10	73	3	11
19	2	12	74	5	5
20	4	5	75	1	2
21	3	4	76	3	4
22	4	1	77	3	4
23	4	2	78	6	2
24	5	2	79	5	2
25	4	3	80	6	5
26	6	4	81	2	11
27	7	7	82	7	3
28	3	2	83	2	3
29	3	4	84	2	4
30	4	4	85	3	5
31	0	6	86	9	3
32	3	3	87	2	5
33	3	2	88	5	5
34	9	2	89	2	1
35	4	1	90	6	6
36	2	8	91	6	4
37	2	3	92	0	1
38	3	0	93	1	2
39	5	4	94	1	0
40	3	0	95	1	0
41	2	4	96	1	3
42	4	8	97	3	3
43	0	3	98	2	1
44	7	3	99	3	1
45	4	1	100	2	3
46	8	2	101	1	3
47	6	1	102	0	1
48	8	5	103	2	2
49	5	3	104	0	
50	4	6	105	2	
51	6	2			
52	6	6			
53	5	10			
54	11	5			
55	3	13			

Table B.21
Nucleus Ambiguus: A15

Nucleolar Counts Per Section

Section Number	A15		Section Number	A15	
	Left	Right		Left	Right
1	13	12	56	2	7
2	12	7	57	10	3
3	15	15	58	8	7
4	11	5	59	4	7
5	14	14	60	5	2
6	9	8	61	1	3
7	8	11	62	3	3
8	8	13	63	6	3
9	11	10	64	4	5
10	11	9	65	9	3
11	13	10	66	4	2
12	2	11	67	1	2
13	9	10	68	4	0
14	5	4	69	4	3
15	9	8	70	6	4
16	5	7	71	6	1
17	5	5	72	1	2
18	5	3	73	2	5
19	3	3	74	2	2
20	8	5	75	2	6
21	3	2	76	1	2
22	1	5	77	3	4
23	2	3	78	4	0
24	2	5	79	1	1
25	2	3	80	2	7
26	3	5	81	1	3
27	2	5	82	1	1
28	8	9	83	4	1
29	5	6	84	4	2
30	3	2	85	6	2
31	6	0	86	1	3
32	2	1	87	2	2
33	5	2	88	2	1
34	6	3	89	3	2
35	1	6	90	2	1
36	5	2	91		1
37	4	6	92		3
38	2	8	93		3
39	3	1	94		2
40	4	4			
41	3	4			
42	4	1			
43	5	7			
44	4	6			
45	6	3			
46	4	6			
47	1	6			
48	6	10			
49	7	6			
50	6	4			
51	4	2			
52	5	7			
53	4	3			
54	8	1			
55	5	4			

Table B.22
Nucleus Ambiguus: A16

Nucleolar Counts Per Section

Section Number	A16		Section Number	A16	
	Left	Right		Left	Right
1	9	6	56	2	3
2	11	10	57	4	8
3	14	15	58	7	7
4	8	10	59	4	4
5	15	10	60	4	9
6	9	14	61	5	7
7	9	8	62	4	3
8	7	9	63	1	1
9	11	10	64	4	2
10	6	7	65	5	2
11	18	5	66	2	9
12	10	5	67	6	3
13	6	6	68	4	1
14	8	6	69	4	3
15	9	7	70	3	5
16	8	7	71	4	1
17	5	6	72	3	5
18	13	9	73	4	5
19	6	5	74	3	3
20	8	8	75	2	8
21	2	6	76	2	2
22	2	2	77	1	6
23	1	3	78	3	1
24	4	0	79	0	7
25	6	2	80	3	2
26	8	1	81	4	1
27	5	4	82	1	3
28	3	0	83	2	5
29	3	7	84	4	2
30	2	2	85	3	1
31	0	5	86	0	5
32	3	4	87	0	2
33	5	6	88	0	3
34	2	2	89	1	0
35	1	1	90	4	0
36	0	1	91		0
37	5	5	92		0
38	2	1	93		2
39	3	1	94		3
40	5	2	95		0
41	7	5	96		1
42	4	5			
43	5	1			
44	11	3			
45	5	7			
46	6	4			
47	8	4			
48	8	12			
49	5	7			
50	4	8			
51	5	2			
52	8	8			
53	7	6			
54	6	4			
55	2	3			

Table B.23
Nucleus Ambiguus: A17

Nucleolar Counts Per Section

Section Number	A17		Section Number	A17	
	Left	Right		Left	Right
1	19	13	56	3	2
2	15	8	57	5	6
3	9	10	58	5	3
4	14	12	59	4	2
5	14	14	60	1	2
6	10	13	61	2	3
7	4	12	62	3	5
8	6	8	63	6	4
9	9	6	64	3	2
10	9	7	65	5	0
11	7	8	66	2	1
12	11	4	67	1	5
13	5	4	68	0	1
14	6	4	69	4	2
15	5	7	70	5	3
16	7	7	71	5	4
17	4	6	72	2	2
18	5	5	73	2	5
19	7	5	74	2	1
20	3	7	75	3	2
21	3	8	76	0	3
22	5	8	77	5	1
23	4	3	78	0	2
24	6	0	79	3	2
25	7	3	80	1	1
26	5	0	81		2
27	6	2	82		1
28	2	1			
29	3	3			
30	1	6			
31	1	3			
32	5	4			
33	5	2			
34	1	2			
35	3	10			
36	5	5			
37	5	10			
38	5	1			
39	2	2			
40	4	6			
41	2	3			
42	5	5			
43	5	5			
44	2	3			
45	4	5			
46	8	5			
47	6	4			
48	8	4			
49	6	10			
50	8	4			
51	11	2			
52	7	11			
53	6	8			
54	7	5			
55	5	3			

Table B.24
Nucleus Ambiguus: A18

Nucleolar Counts Per Section

Section Number	A18		Section Number	A18	
	Left	Right		Left	Right
1	10	10	56	9	5
2	6	5	57	10	9
3	10	6	58	4	7
4	12	6	59	14	6
5	10	13	60	7	12
6	7	8	61	6	6
7	4	13	62	4	9
8	7	8	63	5	5
9	7	7	64	4	6
10	7	8	65	2	8
11	9	9	66	6	5
12	2	8	67	4	6
13	5	12	68	2	3
14	8	6	69	5	8
15	7	8	70	1	2
16	7	8	71	2	1
17	3	5	72	4	2
18	3	3	73	4	2
19	3	9	74	3	4
20	9	8	75	1	1
21	6	6	76	3	0
22	8	6	77	1	2
23	9	2	78	3	2
24	8	4	79	1	2
25	6	5	80	1	2
26	6	3	81	3	6
27	5	5	82	3	2
28	4	2	83	1	2
29	5	5	84	4	4
30	5	9	85	4	3
31	4	3	86	2	3
32	3	1	87	1	0
33	1	4	88	0	3
34	3	2	89	2	1
35	1	6	90	1	2
36	2	4	91	0	3
37	2	2	92	1	2
38	13	5	93		0
39	2	6	94		1
40	6	4			
41	4	4			
42	3	5			
43	3	7			
44	2	2			
45	4	8			
46	5	11			
47	11	4			
48	4	3			
49	0	10			
50	6	7			
51	8	4			
52	9	3			
53	4	8			
54	10	7			
55	5	9			

Table B.25
Nucleus Ambiguus: A19

Nucleolar Counts Per Section

Section Number	A19		Section Number	A19	
	Left	Right		Left	Right
1	6	4	56	9	6
2	5	8	57	5	3
3	11	7	58	8	3
4	12	6	59	4	8
5	6	8	60	7	6
6	12	11	61	5	4
7	10	10	62	4	6
8	15	14	63	5	6
9	9	11	64	8	2
10	5	9	65	8	5
11	7	6	66	1	7
12	8	5	67	2	1
13	9	10	68	3	7
14	6	7	69	5	2
15	4	6	70	8	1
16	4	7	71	6	2
17	6	8	72	4	4
18	5	4	73	6	2
19	7	7	74	1	4
20	5	5	75	3	5
21	7	8	76	2	2
22	4	6	77	5	1
23	4	5	78	2	2
24	4	4	79	1	2
25	9	2	80	2	4
26	5	4	81	3	1
27	3	1	82	3	2
28	4	5	83	3	2
29	3	4	84	0	0
30	5	8	85	4	2
31	1	3	86	3	3
32	1	3	87	2	0
33	6	8	88	1	1
34	5	6	89	1	2
35	2	3	90	2	2
36	5	1	91	4	1
37	8	3	92	2	1
38	1	2	93	2	0
39	4	0	94	5	1
40	6	3	95	0	3
41	3	6	96	3	1
42	3	4	97	1	1
43	1	10	98	0	0
44	0	2	99	1	0
45	1	4	100	1	1
46	4	4	101	0	2
47	6	8	102	2	2
48	4	5			
49	6	2			
50	7	5			
51	5	2			
52	3	5			
53	4	6			
54	4	8			
55	6	2			

Appendix C

This section contains 25 tables, C.1 to C.25. These tables present the nucleolar counts for all specimens used in the investigation of MCD in XII. All ages, except adults, are shown by litter. The following counts were multiplied by the sampling rate to generate estimates of motoneuron numbers in XII as discussed in chapter 7.

Table C.1
Hypoglossal Nucleus: E16, Litter "A"

Nucleolar Counts Per Section

Section Number	E16-A2		E16-A4		E16-A6	
	Left	Right	Left	Right	Left	Right
1	6	7	26	23	5	15
2	29	26	32	31	31	18
3	45	37	50	48	34	39
4	53	58	44	40	31	35
5	56	57	39	49	42	41
6	57	39	44	52	51	43
7	58	55	47	40	46	50
8	44	50	38	48	47	40
9	45	43	43	39	42	53
10	47	46	43	47	46	33
11	50	48	48	46	52	39
12	50	58	42	47	45	50
13	48	56	57	55	47	44
14	54	43	38	37	50	39
15	51	52	50	48	42	54
16	48	50	38	39	48	54
17	53	55	36	47	49	57
18	55	39	49	37	54	39
19	33	37	36	40	39	53
20	45	30	31	38	41	38
21	36	30	30	36	44	39
22	35	21	29	25	39	43
23	19	12	19	22	27	34
24	25		14	10	31	32
25			8	11	19	29
26				2	13	8
27					4	2

Table C.2
Hypoglossal Nucleus: E16, Litter "B"

Nucleolar Counts Per Section

Section Number	E16-B1		E16-B3		E16-B5		E16-B6	
	Left	Right	Left	Right	Left	Right	Left	Right
1	17	8	5	3	4	6	7	5
2	7	19	19	25	14	10	22	15
3	29	30	36	25	21	29	35	23
4	40	42	26	26	30	34	28	26
5	47	36	33	45	29	41	33	32
6	53	56	35	42	40	23	34	33
7	58	46	45	29	29	44	38	35
8	41	47	47	39	28	33	34	52
9	41	48	38	46	39	35	43	44
10	43	55	44	38	35	44	38	30
11	44	50	47	41	40	42	38	47
12	54	43	45	43	37	33	47	35
13	51	41	45	42	41	51	50	39
14	47	27	40	45	49	42	43	31
15	43	50	52	53	39	52	47	43
16	43	53	40	43	49	42	54	38
17	42	47	45	49	35	42	38	35
18	51	45	46	47	45	41	44	44
19	49	39	44	46	39	35	42	39
20	50	45	50	42	38	28	38	41
21	38	28	52	53	36	37	44	39
22	37	36	44	42	36	45	31	40
23	32	41	41	45	28	39	35	35
24	25	34	37	41	40	35	44	33
25	26	22	35	22	38	44	33	39
26	29	24	22	24	39	44	46	31
27	16	11	27	31	38	31	34	26
28	8	10	17	15	27	22	31	26
29	6	11	20	16	33	30	34	30
30	4	2	8	7	19	23	30	29
31			9	7	21	17	37	26
32			3	3	16	17	32	16
33					3	8	10	9
34					5	5	6	3
35					2	2		

Table C.3
Hypoglossal Nucleus: E16, Litter "C"

Nucleolar Counts Per Section

Section Number	E16-C1		E16-C3		E16-C5	
	Left	Right	Left	Right	Left	Right
1	6	3	7	3	5	6
2	15	12	20	10	24	24
3	32	30	27	27	36	33
4	43	38	34	41	39	44
5	51	40	41	38	42	60
6	37	42	44	44	46	49
7	36	48	36	53	61	63
8	44	34	38	40	53	49
9	49	47	52	45	52	60
10	42	53	41	41	57	56
11	51	52	44	49	59	51
12	43	47	47	51	52	54
13	44	36	61	45	64	43
14	43	43	52	46	67	57
15	44	48	48	45	66	53
16	36	37	45	49	61	56
17	37	52	53	54	58	53
18	40	38	43	49	66	69
19	46	48	52	48	70	49
20	48	39	38	41	39	44
21	40	40	40	50	18	18
22	35	32	56	43	2	5
23	28	20	41	28		
24	15	6	31	35		
25			12	30		
26			12	20		
27				9		

Table C.4
Hypoglossal Nucleus: E17, Litter "A"

Nucleolar Counts Per Section

Section Number	E17-A3		E17-A4		E17-A5	
	Left	Right	Left	Right	Left	Right
1	10	12	4	3	11	14
2	38	17	22	11	9	20
3	52	55	23	19	39	33
4	56	50	37	53	36	34
5	45	49	67	46	33	39
6	41	42	46	53	41	46
7	42	44	44	55	33	31
8	44	43	52	58	33	44
9	36	47	46	44	24	30
10	38	40	57	51	43	33
11	46	32	51	46	41	33
12	43	42	51	50	31	45
13	42	42	41	37	38	49
14	44	46	48	46	34	38
15	43	48	50	55	33	23
16	38	37	40	44	26	38
17	41	46	41	43	33	32
18	50	45	43	43	22	27
19	34	33	37	36	18	29
20	26	37	40	48	21	25
21	29	28	44	46	27	22
22	24	27	34	53	18	14
23	22	28	36	30	14	8
24	19	12	37	30	18	12
25	20	21	26	26	16	7
26	13	18	20	21	8	5
27	9	15	26	28	3	
28	5	7	19	15		
29		4	11	10		
30			5	7		
31			5	4		
32			4	3		

Table C.5
Hypoglossal Nucleus: E17, Litter "B"

Nucleolar Counts Per Section

Section Number	E17-B3		E17-B4		E17-B5		E17-B6	
	Left	Right	Left	Right	Left	Right	Left	Right
1	5	10	12	5	10	5	7	5
2	10	12	15	17	14	13	14	5
3	30	31	28	18	16	14	28	28
4	33	28	32	35	45	36	41	37
5	33	30	46	58	41	44	40	37
6	40	26	42	53	48	43	32	31
7	41	52	45	40	39	46	49	40
8	39	55	48	47	34	36	35	44
9	40	38	44	44	39	39	29	32
10	46	39	47	46	48	35	34	30
11	46	47	40	42	37	38	33	32
12	25	39	31	35	47	38	35	33
13	47	42	39	46	45	41	35	32
14	38	47	40	34	44	48	28	36
15	36	39	37	49	53	43	32	33
16	54	39	38	32	36	29	32	37
17	40	44	31	36	42	45	30	23
18	38	39	32	45	45	39	21	20
19	47	43	43	37	43	42	31	22
20	45	31	35	41	32	43	25	26
21	27	29	40	38	40	30	20	22
22	22	27	23	31	31	33	17	19
23	26	18	35	41	25	23	25	16
24	10	21	29	35	26	29	14	11
25	6	9	22	28	25	24	17	11
26	8	9	19	20	18	10	18	6
27	5	7	12	23	10	8	13	10
28	4	2	18	19	4	9		
29			13	15		3		
30			12	6				
31			3	6				

Table C.6
Hypoglossal Nucleus: E17, Litter "C"

Nucleolar Counts Per Section

Section Number	E17-C2		E17-C3		E17-C5	
	Left	Right	Left	Right	Left	Right
1	12	17	5	8	13	5
2	35	29	6	10	7	7
3	41	31	29	25	32	13
4	60	51	40	34	43	31
5	43	34	33	42	38	36
6	55	40	36	40	34	31
7	41	58	46	45	38	32
8	45	49	46	31	35	39
9	53	42	36	45	27	38
10	48	41	35	30	32	35
11	53	58	45	43	38	37
12	53	49	45	40	24	41
13	54	47	49	41	40	46
14	47	49	39	50	43	35
15	52	40	35	41	33	37
16	41	37	48	42	58	31
17	39	43	39	31	38	34
18	49	39	37	38	30	34
19	37	43	30	44	36	37
20	41	34	24	39	40	27
21	26	35	36	27	29	33
22	22	21	25	34	31	43
23	20	22	18	32	28	28
24	14	18	15	23	18	23
25	8	11	10	22	16	11
26	4	6	8	12	9	13
27			8	7	3	8
28			1	2		

Table C.7
Hypoglossal Nucleus: E18, Litter "D"

Nucleolar Counts Per Section

Section Number	E18-D2		E18-D3		E18-D4		E18-D5	
	Left	Right	Left	Right	Left	Right	Left	Right
1	2	3	7	7	2	3	11	10
2	31	11	16	18	11	6	23	22
3	22	33	15	11	21	22	36	26
4	38	25	21	33	39	24	35	26
5	53	47	38	48	44	47	58	42
6	46	45	35	31	45	40	62	36
7	61	38	46	39	46	37	47	54
8	54	51	35	33	34	37	57	38
9	51	45	30	27	36	36	40	43
10	39	43	42	37	32	28	59	56
11	33	38	29	35	33	29	45	41
12	45	45	26	37	37	34	57	52
13	37	49	33	35	37	29	48	47
14	32	38	39	41	25	25	37	40
15	34	45	37	42	31	29	51	46
16	41	29	40	32	34	24	48	40
17	33	39	34	38	32	38	33	47
18	39	29	32	44	36	40	34	42
19	30	25	35	32	31	45	27	32
20	27	28	29	31	38	34	32	22
21	32	31	26	26	27	29	30	31
22	25	24	24	21	27	28	16	10
23	21	34	22	25	29	18	8	7
24	19	29	19	18	14	25	6	7
25	10	25	27	25	14	19	4	8
26	7	14	18	28	8	11	4	4
27	8	12	26	11	6	12	2	
28	2	5	13	11	4	10		
29	3	3	9	2	2	4		
30			2	3		2		
31						1		

Table C.8
Hypoglossal Nucleus: E18, Litter "E"

Nucleolar Counts Per Section

Section Number	E18-E4		E18-E5		E18-E6	
	Left	Right	Left	Right	Left	Right
1	13	8	6	6	1	11
2	19	20	10	20	10	17
3	21	21	10	28	25	18
4	31	24	30	42	28	26
5	44	25	37	33	37	35
6	43	35	35	35	31	35
7	41	35	38	43	23	36
8	34	43	35	40	33	31
9	33	38	33	38	38	37
10	46	35	43	45	28	23
11	33	49	34	46	29	39
12	35	38	38	30	40	32
13	46	38	41	36	28	36
14	39	50	32	43	32	28
15	37	45	47	42	25	31
16	31	40	39	43	33	34
17	32	37	47	40	32	36
18	33	43	34	34	24	34
19	35	37	37	39	33	36
20	39	35	32	38	21	31
21	33	40	39	41	25	25
22	21	29	32	39	21	30
23	26	28	30	31	25	21
24	23	23	24	26	29	20
25	13	15	21	20	14	16
26	10	14	19	17	10	21
27	15	4	21	16	7	20
28	5	4	10	18	6	7
29	1	4	10	5	7	3
30	1		7	3	4	
31			1	4	1	
32			4			

Table C.9
Hypoglossal Nucleus: E18, Litter "F"

Nucleolar Counts Per Section

Section Number	E18-F3		E18-F4		E18-F5	
	Left	Right	Left	Right	Left	Right
1	6	6	9	3	4	10
2	7	19	14	10	19	15
3	27	22	29	37	25	28
4	39	36	43	38	40	43
5	59	49	41	53	52	42
6	47	41	48	35	41	41
7	47	54	42	48	42	40
8	39	41	38	43	55	53
9	45	45	45	33	43	46
10	39	45	30	39	43	49
11	35	42	41	32	51	33
12	43	32	40	34	36	40
13	40	39	40	38	32	34
14	54	46	35	45	37	42
15	43	33	43	40	42	44
16	33	31	46	34	48	47
17	45	40	41	42	32	34
18	32	33	45	34	36	45
19	31	41	32	30	40	41
20	35	41	37	42	39	39
21	39	43	23	22	45	27
22	26	34	27	21	24	30
23	26	28	21	24	15	18
24	19	22	28	28	19	19
25	17	17	17	29	10	9
26	16	19	17	14	13	14
27	8	12	22	12		6
28	7	4	9	8		6
29	1		7	7		4
30			6	6		
31			1			

Table C.10
Hypoglossal Nucleus: E19, Litter "A"

Nucleolar Counts Per Section

Section Number	E19-A4		E19-A5		E19-A6	
	Left	Right	Left	Right	Left	Right
1	5	7	9	5	2	12
2	11	13	15	20	10	22
3	22	20	11	23	12	20
4	27	34	33	29	32	32
5	42	34	34	35	25	41
6	41	35	36	35	28	31
7	35	32	31	30	32	35
8	38	24	28	26	35	28
9	35	38	27	28	38	32
10	44	41	35	29	39	40
11	45	37	30	29	35	31
12	50	43	31	35	44	26
13	35	39	30	30	26	35
14	29	39	37	40	37	22
15	39	32	40	27	31	36
16	33	31	26	25	37	32
17	31	29	23	26	46	36
18	36	31	25	27	39	28
19	36	20	29	30	43	32
20	17	20	20	23	24	41
21	13	14	28	22	34	31
22	17	16	19	18	27	33
23	14	9	18	22	27	26
24	4	5	9	9	26	18
25	7	2	10	7	17	16
26		4	11	1	9	12
27			5	2	9	13
28					8	4
29					1	1

Table C.11
Hypoglossal Nucleus: E19, Litter "B"

Nucleolar Counts Per Section

Section Number	E19-B2		E19-B3		E19-B4		E19-B5	
	Left	Right	Left	Right	Left	Right	Left	Right
1	13	14	3	13	5	14	12	10
2	23	25	18	18	17	10	19	19
3	12	15	20	20	19	13	21	16
4	17	19	25	40	22	13	32	28
5	22	34	36	37	20	24	33	29
6	36	35	43	34	25	25	32	32
7	23	28	35	37	21	29	33	23
8	30	34	32	32	28	23	33	32
9	27	26	37	34	29	19	24	26
10	27	30	33	35	22	26	26	29
11	40	24	42	32	28	25	30	31
12	28	52	49	41	20	26	35	22
13	27	28	37	35	27	18	40	31
14	27	29	30	34	33	24	24	32
15	34	33	33	31	27	32	26	30
16	37	39	40	36	24	33	29	22
17	33	24	42	30	32	19	33	33
18	23	24	33	30	24	20	29	25
19	19	30	28	40	20	18	18	36
20	26	25	30	31	23	15	25	28
21	26	28	21	22	18	14	24	24
22	17	14	29	28	21	19	23	21
23	21	16	27	19	13	9	16	18
24	26	18	26	18	20	22	15	16
25	15	9	29	24	6	9	11	14
26	11	7	11	11	4	7	3	15
27	4	2	11	6	3	5	6	7
28			8	3			3	8
29			4	2				1
30			2					
31			2					
32			1					

Table C.12
Hypoglossal Nucleus: E19, Litter "C"

Nucleolar Counts Per Section

Section Number	E19-C1		E19-C3		E19-C5	
	Left	Right	Left	Right	Left	Right
1	5	5	5	4	8	9
2	26	24	19	17	16	8
3	27	19	15	24	21	20
4	37	29	34	25	40	32
5	47	40	38	40	40	27
6	44	39	36	39	27	38
7	38	36	42	36	38	33
8	34	32	40	36	36	32
9	40	41	37	28	38	34
10	35	40	35	38	42	31
11	30	37	31	27	30	32
12	41	26	39	30	39	31
13	34	39	31	26	35	37
14	35	37	38	42	26	37
15	35	33	27	31	26	30
16	31	33	33	44	42	34
17	32	31	31	34	30	36
18	38	44	27	29	31	26
19	32	37	33	30	28	22
20	25	34	26	27	28	29
21	19	22	21	28	30	28
22	21	25	21	23	27	25
23	22	23	30	29	26	27
24	19	17	17	31	18	11
25	16	17	13	25	5	12
26	8	11	9	24	5	11
27	7	18	10	12	4	2
28	8	8	7	4		
29		2	3			

Table C.13
Hypoglossal Nucleus: P1, Litter "E"

Nucleolar Counts Per Section

Section Number	P1-E1		P1-E2		P1-E3	
	Left	Right	Left	Right	Left	Right
1	4	5	3	5	6	8
2	16	11	12	10	12	11
3	15	27	18	16	17	16
4	17	21	20	17	23	25
5	28	20	27	25	35	26
6	35	32	34	28	30	33
7	21	33	28	21	45	37
8	27	27	31	29	38	39
9	26	35	31	29	36	41
10	29	31	36	30	34	30
11	30	27	38	22	30	25
12	22	35	41	27	39	33
13	35	25	30	32	31	32
14	27	24	33	23	33	34
15	38	30	29	29	33	29
16	30	34	30	31	31	23
17	30	37	28	24	32	28
18	26	31	27	23	37	32
19	34	33	29	35	44	22
20	37	33	23	26	29	32
21	28	18	26	27	30	25
22	27	24	28	28	20	21
23	28	17	23	26	23	19
24	20	19	20	18	23	17
25	21	23	22	18	12	13
26	18	17	16	11	13	9
27	13	16	9	8	14	8
28	13	14	3	2	4	2
29	3	4		1		
30		3				

Table C.14
Hypoglossal Nucleus: P1, Litter "F"

Nucleolar Counts Per Section

Section Number	P1-F1		P1-F2		P1-F3		P1-F4	
	Left	Right	Left	Right	Left	Right	Left	Right
1	9	8	3	4	5	3	4	3
2	13	25	10	16	8	14	15	19
3	16	26	29	24	25	16	21	24
4	28	29	31	26	32	19	25	26
5	26	28	42	35	32	27	46	34
6	32	30	37	32	40	19	33	46
7	36	32	34	27	29	22	37	28
8	32	35	37	32	24	29	38	32
9	29	37	36	37	27	17	41	35
10	36	30	25	40	31	34	37	50
11	31	34	26	39	23	28	42	32
12	30	30	30	34	31	23	36	37
13	37	35	33	34	34	31	55	34
14	37	25	39	24	18	29	43	39
15	31	30	32	39	26	26	30	36
16	36	25	33	37	32	24	37	34
17	28	30	37	34	28	25	31	29
18	30	26	32	28	32	40	27	37
19	23	20	31	26	33	31	28	30
20	20	15	21	33	24	23	22	31
21	22	15	25	24	23	23	15	16
22	14	10	20	19	30	26	8	12
23	12	6	15	10	23	24	5	2
24	6	4	4	4	16	25		1
25		1	5	1	18	12		
26			1		18	13		
27					9	2		
28					4			

Table C.15
Hypoglossal Nucleus: P1, Litter "G"

Nucleolar Counts Per Section

Section Number	P1-G5		P1-G6		P1-G7	
	Left	Right	Left	Right	Left	Right
1	10	3	4	5	8	9
2	22	38	18	19	25	21
3	33	32	22	40	33	36
4	46	27	41	33	27	37
5	37	38	37	34	49	51
6	34	41	47	44	43	39
7	45	43	44	37	52	41
8	51	52	49	45	45	49
9	49	45	52	46	47	45
10	42	40	39	54	52	52
11	37	40	40	47	51	39
12	53	42	40	52	45	54
13	44	46	48	44	50	46
14	40	35	40	45	34	34
15	31	34	39	43	42	47
16	37	27	38	30	34	32
17	26	27	32	30	20	19
18	19	25	28	22	15	9
19	12	17	11	12	4	
20	4	6	3	5		

Table C.16
Hypoglossal Nucleus: A8
Nucleolar Counts Per Section

Section Number	A8		Section Number	A8	
	Left	Right		Left	Right
1	14	10	51	12	11
2	9	18	52	10	11
3	19	20	53	10	11
4	16	19	54	8	10
5	12	18	55	15	15
6	15	20	56	8	7
7	16	17	57	10	6
8	16	10	58	8	8
9	25	16	59	12	10
10	15	19	60	15	11
11	16	17	61	14	11
12	20	14	62	7	8
13	17	17	63	10	6
14	14	27	64	6	4
15	25	14	65	5	3
16	21	21	66	3	2
17	21	26	67	4	4
18	23	19			
19	23	21			
20	25	21			
21	23	22			
22	20	22			
23	20	18			
24	24	19			
25	18	20			
26	16	17			
27	19	28			
28	26	21			
29	28	23			
30	26	19			
31	28	30			
32	20	28			
33	28	27			
34	34	27			
35	27	27			
36	20	27			
37	24	29			
38	28	20			
39	27	23			
40	30	31			
41	31	24			
42	28	24			
43	28	24			
44	24	23			
45	12	16			
46	15	13			
47	15	14			
48	20	19			
49	20	19			
50	17	13			

Table C.17
Hypoglossal Nucleus: A9

Nucleolar Counts Per Section

Section Number	A9		Section Number	A9	
	Left	Right		Left	Right
1	11	9	51	20	18
2	5	8	52	16	14
3	11	10	53	20	12
4	11	14	54	15	18
5	18	14	55	16	10
6	15	13	56	18	14
7	14	13	57	16	13
8	13	16	58	14	12
9	17	14	59	9	8
10	17	19	60	19	12
11	22	20	61	15	10
12	8	20	62	8	12
13	13	12	63	12	6
14	15	16	64	10	16
15	20	13	65	14	4
16	11	15	66	11	9
17	14	16	67	7	8
18	16	12	68	11	4
19	16	18	69	8	5
20	19	16	70	7	9
21	19	19	71	4	7
22	17	15			
23	21	15			
24	13	15			
25	20	16			
26	15	12			
27	17	14			
28	16	21			
29	14	13			
30	18	18			
31	16	17			
32	19	18			
33	16	23			
34	12	18			
35	18	13			
36	21	16			
37	26	14			
38	14	16			
39	13	18			
40	19	19			
41	21	22			
42	16	19			
43	15	18			
44	23	31			
45	18	23			
46	20	21			
47	20	22			
48	17	12			
49	25	21			
50	23	17			

Table C.18
Hypoglossal Nucleus: A10
Nucleolar Counts Per Section

Section Number	A10		Section Number	A10	
	Left	Right		Left	Right
1	5	6	51	13	19
2	11	11	52	20	21
3	17	16	53	19	16
4	13	7	54	13	17
5	16	14	55	14	19
6	10	12	56	17	18
7	13	11	57	18	20
8	14	12	58	17	14
9	13	9	59	20	13
10	12	18	60	15	9
11	9	16	61	9	10
12	14	24	62	13	8
13	14	15	63	8	12
14	7	17	64	11	11
15	10	10	65	15	12
16	11	9	66	4	11
17	15	8	67	10	7
18	12	8	68	4	5
19	9	16	69	8	4
20	10	10	70	10	6
21	10	11	71	3	4
22	9	7			
23	10	16			
24	11	10			
25	16	18			
26	17	17			
27	14	22			
28	13	15			
29	18	17			
30	22	11			
31	16	17			
32	16	16			
33	19	21			
34	15	18			
35	21	16			
36	25	16			
37	15	26			
38	17	17			
39	19	30			
40	24	22			
41	25	18			
42	20	26			
43	22	24			
44	20	17			
45	20	16			
46	21	17			
47	16	16			
48	21	15			
49	19	25			
50	21	15			

Table C.19
Hypoglossal Nucleus: A11

Nucleolar Counts Per Section

Section Number	A11		Section Number	A11	
	Left	Right		Left	Right
1	10	11	51	14	16
2	8	11	52	9	10
3	10	10	53	11	16
4	12	11	54	12	17
5	13	12	55	15	7
6	11	12	56	12	19
7	10	11	57	20	14
8	10	9	58	12	9
9	10	17	59	15	13
10	17	18	60	14	10
11	9	16	61	9	10
12	12	11	62	12	11
13	15	12	63	12	11
14	21	18	64	12	7
15	14	16	65	9	11
16	17	21	66	10	10
17	17	20	67	9	8
18	19	23	68	8	13
19	12	22	69	15	14
20	16	20	70	12	9
21	11	16	71	10	6
22	12	14	72	4	
23	12	14			
24	14	17			
25	19	21			
26	14	13			
27	10	20			
28	21	15			
29	13	13			
30	16	16			
31	21	14			
32	18	16			
33	9	15			
34	16	15			
35	19	17			
36	16	19			
37	24	19			
38	17	12			
39	15	20			
40	23	19			
41	25	18			
42	23	26			
43	22	26			
44	21	20			
45	15	17			
46	20	17			
47	18	14			
48	15	15			
49	14	16			
50	12	18			

Table C.20
Hypoglossal Nucleus: A12

Nucleolar Counts Per Section

Section Number	A12		Section Number	A12	
	Left	Right		Left	Right
1	3	2	51	18	25
2	5	5	52	19	12
3	3	9	53	18	22
4	8	7	54	17	16
5	5	7	55	16	12
6	9	8	56	15	16
7	11	12	57	20	13
8	8	8	58	16	11
9	10	8	59	8	17
10	13	18	60	14	11
11	14	14	61	16	11
12	19	13	62	17	9
13	10	15	63	8	10
14	11	17	64	7	10
15	12	13	65	7	3
16	14	13	66	7	9
17	24	12	67	10	8
18	13	10	68	9	1
19	11	14	69	6	5
20	12	13	70	5	9
21	13	14	71	8	5
22	16	13	72	6	8
23	10	16	73	10	7
24	17	14	74	5	6
25	11	18	75	5	4
26	10	13	76	7	7
27	14	14	77	2	3
28	17	15	78	2	5
29	20	9	79	2	6
30	11	15	80	1	1
31	17	13	81	1	
32	17	12			
33	14	16			
34	20	17			
35	12	24			
36	11	15			
37	13	21			
38	14	19			
39	19	15			
40	14	16			
41	13	17			
42	20	24			
43	22	28			
44	19	24			
45	19	18			
46	14	11			
47	12	14			
48	8	15			
49	16	14			
50	19	18			

Table C.21
Hypoglossal Nucleus: A13

Nucleolar Counts Per Section

Section Number	A13		Section Number	A13	
	Left	Right		Left	Right
1	1	3	51	27	14
2	6	4	52	14	14
3	3	6	53	14	16
4	4	5	54	18	16
5	5	6	55	17	19
6	7	6	56	19	23
7	5	5	57	13	20
8	18	11	58	17	19
9	6	8	59	22	20
10	9	12	60	19	13
11	8	7	61	22	20
12	10	13	62	13	13
13	9	12	63	16	17
14	8	12	64	16	21
15	10	13	65	17	19
16	9	11	66	9	12
17	7	7	67	21	24
18	10	10	68	18	14
19	12	9	69	21	21
20	10	12	70	19	14
21	9	5	71	14	5
22	5	6	72	20	12
23	5	13	73	15	6
24	17	16	74	10	11
25	13	10	75	17	15
26	13	20	76	10	11
27	18	12	77	13	13
28	24	11	78	12	10
29	13	16	79	11	8
30	21	14	80	8	9
31	21	12	81	11	5
32	25	18	82	14	9
33	18	24	83	5	5
34	18	13	84	10	6
35	14	15	85	6	9
36	17	20	86	12	6
37	11	18	87	9	3
38	20	14	88	6	2
39	19	18	89	5	1
40	22	18	90		4
41	15	19	91		2
42	10	15	92		4
43	12	19			
44	14	17			
45	15	17			
46	13	18			
47	14	25			
48	15	25			
49	18	17			
50	22	19			

Table C.22
Hypoglossal Nucleus: A15

Nucleolar Counts Per Section

Section Number	A15		Section Number	A15	
	Left	Right		Left	Right
1	4	4	51	17	21
2	5	8	52	26	15
3	3	6	53	15	9
4	8	12	54	14	18
5	15	12	55	16	17
6	10	3	56	19	17
7	11	6	57	18	15
8	6	5	58	14	18
9	10	15	59	15	19
10	8	11	60	21	21
11	12	17	61	18	16
12	12	16	62	13	10
13	11	14	63	16	5
14	11	15	64	9	11
15	17	15	65	11	9
16	16	20	66	12	3
17	18	24	67	9	5
18	18	20	68	13	7
19	20	22	69	13	6
20	20	15	70	7	3
21	18	14	71	10	
22	17	20	72	5	3
23	14	15	73	2	2
24	21	14	74	1	
25	14	13			
26	12	16			
27	18	14			
28	13	14			
29	25	17			
30	11	15			
31	14	15			
32	17	18			
33	19	21			
34	21	24			
35	23	24			
36	25	15			
37	18	22			
38	22	26			
39	22	20			
40	23	27			
41	21	22			
42	15	13			
43	17	19			
44	23	17			
45	26	15			
46	26	20			
47	16	25			
48	18	19			
49	18	24			
50	19	22			

Table C.23
Hypoglossal Nucleus: A16

Nucleolar Counts Per Section

Section Number	A16		Section Number	A16	
	Left	Right		Left	Right
1	5	8	51	16	19
2	7	12	52	20	20
3	13	10	53	26	23
4	7	6	54	15	14
5	16	10	55	17	21
6	14	11	56	22	21
7	13	13	57	21	17
8	10	14	58	20	17
9	17	11	59	22	16
10	11	12	60	19	11
11	15	17	61	13	10
12	8	14	62	16	14
13	21	21	63	14	24
14	22	24	64	13	19
15	22	27	65	11	18
16	16	23	66	15	18
17	23	21	67	9	15
18	16	23	68	8	10
19	23	15	69	9	11
20	23	17	70	9	9
21	14	18	71	9	11
22	24	18	72	8	8
23	19	22	73	10	12
24	14	25	74	9	8
25	15	26	75	6	5
26	21	19	76	7	7
27	20	26	77	6	2
28	26	21	78	5	1
29	23	16	79	2	2
30	22	17	80	3	0
31	14	17	81	2	3
32	28	19	82	1	1
33	22	18	83	2	2
34	17	22	84	3	1
35	27	16	85	0	1
36	17	19	86	4	1
37	12	19	87	0	1
38	20	17	88	1	1
39	19	16			
40	19	26			
41	18	26			
42	21	20			
43	19	18			
44	21	19			
45	16	24			
46	24	22			
47	20	22			
48	15	20			
49	23	21			
50	15	23			

Table C.24
Hypoglossal Nucleus: A17
Nucleolar Counts Per Section

Section Number	A17		Section Number	A17	
	Left	Right		Left	Right
1	3	4	51	22	20
2	6	3	52	19	18
3	3	10	53	20	16
4	7	9	54	14	12
5	6	11	55	14	15
6	2	14	56	20	11
7	2	8	57	17	11
8	8	8	58	17	11
9	7	8	59	11	14
10	9	17	60	15	13
11	18	16	61	13	9
12	13	18	62	16	9
13	10	18	63	13	13
14	9	16	64	20	12
15	16	10	65	5	14
16	11	13	66	9	8
17	5	17	67	12	5
18	17	15	68	10	10
19	7	17	69	6	8
20	13	20	70	8	7
21	13	13	71	4	5
22	17	18	72	8	2
23	16	14	73	6	1
24	18	14	74	5	4
25	17	18	75	3	4
26	19	8	76	4	0
27	19	12	77	5	2
28	10	13	78	2	3
29	16	14	79	3	1
30	16	15	80	2	1
31	23	16	81	6	1
32	20	20	82	2	
33	13	11			
34	14	16			
35	20	22			
36	9	18			
37	17	20			
38	17	15			
39	16	18			
40	15	19			
41	15	15			
42	13	12			
43	16	18			
44	17	19			
45	19	14			
46	17	16			
47	17	17			
48	17	13			
49	15	14			
50	21	14			

Table C.25
Hypoglossal Nucleus: A18

Nucleolar Counts Per Section

Section Number	A18		Section Number	A18	
	Left	Right		Left	Right
1	3	3	51	20	18
2	3	5	52	20	19
3	2	2	53	22	12
4	7	9	54	22	19
5	3	12	55	14	19
6	5	5	56	15	12
7	6	6	57	17	17
8	8	13	58	14	12
9	16	19	59	14	19
10	11	12	60	13	14
11	10	14	61	16	16
12	15	17	62	18	11
13	23	12	63	14	13
14	14	12	64	10	12
15	17	19	65	12	8
16	11	12	66	9	12
17	18	17	67	15	11
18	13	14	68	7	13
19	19	19	69	7	9
20	13	18	70	11	12
21	17	14	71	8	6
22	14	17	72	6	9
23	14	23	73	8	9
24	15	15	74	4	5
25	13	14	75	6	1
26	14	14	76	5	1
27	16	12	77	3	4
28	17	18	78	6	4
29	23	18	79	2	3
30	15	17	80	0	2
31	21	15	81	2	2
32	17	22	82	2	0
33	13	17	83	0	0
34	19	17	84	1	1
35	10	23			
36	24	13			
37	23	17			
38	17	14			
39	17	17			
40	22	21			
41	19	18			
42	13	23			
43	28	23			
44	25	17			
45	21	19			
46	24	25			
47	26	24			
48	14	21			
49	30	20			
50	18	18			

Appendix D

Complete Methodology for the Assessment of MCD

Prenatal Tissue

- Anesthesia of the pregnant rat by intramuscular injection of ketamine and rompun (0.0006 cc/g each). I.M. injection of ketamine was used as opposed to the usual intraperitoneal route to avoid damage to offspring in the uterine horns which occasionally overlaid the abdominal viscera.
- Perfusion of the mother with 0.1M PBS, pH=7.4 for 30 seconds at high pressure and flow rate. This clears the vasculature of blood which clots when in contact with Carnoy's fixative.
- Perfusion with Carnoy's fixative (ethanol:chloroform:acetic acid; 6:3:1) at high pressure and flow rate until the uterine walls are blanched by fixative. This typically takes no more than 5 minutes.
- Removal of uterus, incision of uterine walls between each sac, and immersion in Carnoy's during subsequent processing of individual prenatal rats.
- Removal of a uterine sac from the fixative and dissection of the fetus from the amniotic and chorionic membranes.
- Measurements to control for fetal age are taken, i.e., weight and crown-rump length.
- Cranium incised along fronto-parietal suture and then excision of parietal and occipital cartilage. Incision of membrane over fourth ventricle to allow exposure of brainstem to fixative.
- Immersion in Carnoy's for 30 minutes with agitation.
- Dissection of brain and brainstem to expose ventral surface to fix.
- Immersion fixation for additional 2.5 hours.
- Complete removal of brain and brainstem. A transverse incision made across the forebrain to provide a "base" for proper orientation of the brainstem during wax embedding.
- Immersion of the brainstem in 100% ethanol for 2 hours.

- Immersion of the brainstem in xylene until cleared (approximately 2 hours).
- Immersion of brainstem in paraffin at 55-57°C under vacuum overnight.
- Embedding in paraffin block oriented so as to cut from rostral to caudal.
- Sectioning of brainstem at 8µm collecting two sets of every fifth section and 10 sections per slide for staining. This protocol (two sets, every fifth, 10 per slide) establishes a collection pattern of i_0 , i_3 , i_5 , i_8 , i_{10} , i_{13} , i_{15} , i_{18} , i_{20} , and i_{23} for each slide. Sections are collected on 45°C water bath containing approximately 200ml of 5% gelatin. After each slide is cut it is placed in a 57°C oven for 10 minutes to adhere sections to slide.
- Deparaffinization and rehydration through graded alcohols.
- Staining by standard hematoxylin and eosin protocol:
 - 15 minutes Harris' Type hematoxylin
 - Rinse in dH₂O
 - Few dips in acid alcohol (200ml 70% EtOH, 2ml concentrated HCL)
 - Rinse in dH₂O
 - 20 seconds in 1% lithium carbonate
 - 20 minutes rinse in running H₂O
 - 25 seconds 200ml eosin (1 part stock, 3 parts 80% EtOH, 1ml glacial acetic acid) Eosin stock: 1g eosin, 20ml dH₂O, 80ml 95% EtOH
 - 2 minutes 95% EtOH
 - 2 minutes 100% EtOH
 - 2 minutes xylene (2x)
 - Coverslip with permount
- Examination under the microscope to determine rostral-most section containing the nucleus under study.
- Counts made from rostral-most section caudally on both the left and right sides until the pyramidal decussation. Only one set is counted which means counting

every other section on a slide due to our sampling procedure. That is, sections i_0 , i_5 , i_{10} , i_{15} , and i_{20} or sections i_3 , i_8 , i_{13} , i_{18} , and i_{23} counted on each slide, exclusive.

- Raw counts totaled and multiplied by five to give total number of cells in the nucleus. The left and right sides averaged to give counts for that animal. 10 animals per age representing 3 different litters studied. All mathematical manipulation of data done on Microsoft Excel for Windows and double checked by hand.

Postnatal Tissue: P1

- Newborns removed from the mother as soon after birth as possible.
- Anesthesia carried out by hypothermia, i.e., newborns put in ice for several minutes.
- Newborns weighed. Crown-rump length is inappropriate in postnatal specimens, as well as impossible due to mature morphology of cervical extension.
- Newborns transcardially perfused using butterfly needle and 10 ml syringe filled with 4% paraformaldehyde in 0.1M PBS, pH=7.4.
- Brains and brainstems dissected out.
- Immersion fixation for one week.
- Removal of dura and transverse sectioning of forebrain to provide a "base" for wax embedding.
- Immersion of the brainstem in 70% ethanol for 2 hours.
- Immersion of the brainstem in 95% ethanol for 2 hours.
- Immersion of the brainstem in 100% ethanol for 2 hours.
- Immersion of the brainstem in xylene until cleared (approximately 2 hours).
- Immersion of brainstem in paraffin at 55-57°C under vacuum overnight.
- Embedding in paraffin block oriented so as to cut from rostral to caudal.

- Sectioning of brainstem at 8µm collecting two sets of every fifth section and 10 sections per slide for staining. See prenatal sectioning for protocol.
- Deparaffinization and rehydration through graded alcohols.
- Staining by standard Nissl protocol:
 - 5.5 minutes in cresylviolet solution (2.5g cresylecht violet, 1.5ml glacial acetic acid, 500ml dH₂O, filtered)
 - Rinse in dH₂O (3x)
 - 1 minute 70% EtOH
 - Differentiation in 200ml 95% EtOH, 1ml glacial acetic acid (approximately 30 seconds)
 - 1 minute 95% EtOH
 - 2 minutes 100% EtOH (2x)
 - 2 minutes xylene (2x)
 - Coverslip with permount
- Examination under the microscope to determine rostral-most section containing the nucleus under study.
- Counts made from rostral-most section caudally on both the left and right sides until the pyramidal decussation. Only one set of every fifth section counted (see prenatal tissue).
- Raw counts totaled and multiplied by five to give total number of cells in the nucleus. The left and right sides averaged to give counts for that animal. 10 animals representing 3 different litters studied. All mathematical manipulation of data done on Microsoft Excel for Windows and double checked by hand.

Postnatal Tissue: Adults

- Anesthesia by intraperitoneal injection of ketamine (0.0006 cc/g) followed by equal amount of intramuscular rompun.

- Perfusion with 0.1M PBS, pH=7.4 for 30 seconds at high pressure and flow rate.
- Perfusion with 4% paraformaldehyde (10% formalin) in 0.1M PBS, pH=7.4.
- Brain and brainstem dissected out.
- Immersion fixation for one week.
- Removal of dura and cerebellum and transverse sectioning at inferior colliculus to provide a "base" for wax embedding.
- Immersion of the brainstem in 70% ethanol for 2 hours.
- Immersion of the brainstem in 95% ethanol for 2 hours.
- Immersion of the brainstem in 100% ethanol for 2 hours.
- Immersion of the brainstem in xylene until cleared (overnight).
- Immersion of brainstem in paraffin at 55-57°C under vacuum overnight.
- Embedding in paraffin block oriented so as to cut from rostral to caudal.
- Sectioning of brainstem at 10µm collecting one set of every third section and 10 sections per slide for staining.
- Deparaffinization and rehydration through graded alcohols.
- Staining by standard Nissl protocol:
 - 5.5 minutes in cresylviolet solution (2.5g cresylecht violet, 1.5ml glacial acetic acid, 500ml dH₂O, filtered)
 - Rinse in dH₂O (3x)
 - 1 minute 70% EtOH
 - Differentiation in 200ml 95% EtOH, 1ml glacial acetic acid (approximately 30 seconds)
 - 1 minute 95% EtOH
 - 2 minutes 100% EtOH (2x)
 - 2 minutes xylene (2x)
 - Coverslip with permount

- Examination under the microscope to determine rostral-most section containing the nucleus under study.
- Counts made from rostral-most section caudally on both the left and right sides until the pyramidal decussation. Every section on slide counted.
- Raw counts totaled and multiplied by three to give total number of cells in the nucleus. The left and right sides averaged to give counts for that animal. 10 animals per nucleus studied. All mathematical manipulation of data done on Microsoft Excel for Windows and double checked by hand.

Appendix E

Complete Methodology for the Investigation of Nucleus Formation and GABAergic Innervation

Complete Methodology for the Investigation of Nucleus Formation

- Anesthesia of pregnant rat by intramuscular injection of ketamine and rompun (0.0006 cc/g each). I.M. injection of ketamine was used as opposed to the usual intraperitoneal route to avoid damage to offspring in the uterine horns which occasionally overlaid the abdominal viscera.
- Incision of the lateral abdominal wall and extrusion of the uterine horn onto sterile gauze soaked with normal saline.
- Incision of uterine and amniotic sac to expose prenatal rat and umbilical vessels.
- Insertion of 30g needle into the umbilical vein. Perfusion with 10ml of 4% paraformaldehyde in 0.1M PBS, pH=7.4. Animal perfused slowly to avoid rupture of the prenatal heart. Umbilical artery cut to provide outlet for flow and to reduce the high perfusion pressure.
- Umbilical vessels cut and prenatal rat removed from mother.
- Incision of lambdoid sutures and removal of occipital bone to expose dorsal brainstem to subsequent immersion fixation. Immersion fixed until used in DiI trace. Some specimens were traced after one week and others after several months. There was no apparent difference in tracing quality.
- Dissection of brain and brainstem. Chondrocranium "pinched" with forceps to free vagus and glossopharyngeal nerves. Hypoglossal nerves could not be isolated but the chondrocranium was carefully removed to prevent tearing of hypoglossal rootlets.
- Approximately 5µl of fast DiI placed on a coverslip and allowed to dry (approximately 30 seconds). Resultant "paste" scraped onto the tip of a number 11 scalpel blade.

- Application of DiI paste to vagus and glossopharyngeal nerves (nucleus ambiguus study) or ventral surface of lower medulla (hypoglossal nucleus study). Paste allowed to dry for several minutes to promote adherence to tissue.
- Immersion of brainstem in fixative at 37°C for approximately 1 week.
- Embedding of brainstem in 4% low melting agarose: agarose dissolved in boiling water and allowed to cool slightly; brainstem placed in small weighing dish and covered in agarose; agarose allowed to gel and then cut into a block for vibratome sectioning.
- Sectioning of brainstem at 40µm on vibratome in ice-cold 0.1M phosphate buffer with slow knife speed and high vibration rate. Sections mounted on slides and allowed to dry.
- Immediate visualization of trace under fluorescent microscopy (Texas Red optics). Results photographed for documentation as sections can not be coverslipped.

Complete Methodology for the Investigation of GABAergic Innervation

- Anesthesia of pregnant rat by intramuscular injection of ketamine and rompun (0.0006 cc/g each). I.M. injection of ketamine was used as opposed to the usual intraperitoneal route to avoid damage to offspring in the uterine horns which occasionally overlaid the abdominal viscera.
- Incision of the lateral abdominal wall and extrusion of the uterine horn onto sterile gauze soaked with normal saline.
- Incision of uterine and amniotic sac to expose prenatal rat and umbilical vessels.
- Insertion of 30g needle into the umbilical vein. Perfusion with 10ml of 4% paraformaldehyde / 0.3% gluteraldehyde in 0.1M PBS, pH=7.2. Animal

perfused slowly to avoid rupture of the prenatal heart. Umbilical artery cut to provide outlet for flow and to reduce the high perfusion pressure.

- Umbilical vessels cut and prenatal rat removed from mother.
- Incision of lambdoid and sagittal sutures and removal of entire brainstem for subsequent immersion fixation. Immersion fixed for 2 hours and transferred to 0.1M phosphate buffer (PBS) for 1 hour wash.
- Embedding of brainstem in 4% low melting agarose: agarose dissolved in boiling water and allowed to cool slightly; brainstem placed in small weighing dish and covered in agarose; agarose allowed to gel and then cut into a block for vibratome sectioning.
- Sectioning of brainstem at 40µm on vibratome in ice-cold 0.1M PBS with slow knife speed and high vibration rate. Sections collected in scintillation vials containing ice-cold phosphate buffer.
- Immunocytochemical Staining Protocol:
 - Incubation in 0.3% H₂O₂ for 30 minutes to block endogenous peroxidase activity.
 - Wash 2 x 10 minutes in 0.1M PBS.
 - Incubation in 0.4% Triton X-100 for 1 hour.
 - Wash 2 x 5 minutes in 0.1M PBS.
 - Incubation in blocking serum (1.5% goat serum) for 30 minutes.
 - Wash 2 x 5 minutes in 0.1M PBS.
 - Incubation in primary antibody at a dilution of 1:7000 for 3 days at 4°C.
 - Wash 3 x 10 minutes in 0.1M PBS.
 - Incubation in secondary biotinylated antibody for 1 hour.
 - Wash 3 x 10 minutes in 0.1M PBS.
 - Incubation in avidin-biotin complex (ABC) reagent for 1 hour.
 - Wash 3 x 10 minutes in 0.1M PBS.

Incubation in substrate solution (0.05% DAB / 0.03% H₂O₂) for 5 minutes.

Wash 3 x 10 minutes in 0.1M PBS.

- **Mount sections on chrome alum subbed slides.**
- **Apply one drop of DAPI solution (2µg/ml) to each section to stain nuclei.**
- **Allow sections to dry and then coverslip with PermaFluor mounting media.**

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