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Characterization of the developing facial and hypoglossal motor systems in the neonatal Brazilian opossum

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**Characterization of the developing facial and hypoglossal
motor systems in the neonatal Brazilian opossum**

by

Jack Jeffrey Swanson

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

Co-majors: Veterinary Anatomy; Neuroscience

Major Professors: Carol D. Jacobson and M. Cathleen Kuehl-Kovarik

Iowa State University

Ames, Iowa

1997

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For the Graduate College

DEDICATION

In honor of my new son Jack Dale Swanson (3/26/97);
in memory of Dale E. Boyd who inspired my journalism skills;
thank you to my family and parents Jack and Mary Jo Swanson;
and to my ever-supportive wife Lori A. Swanson, for making all of this possible.

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ABSTRACT

Aspects of development of the facial and hypoglossal motor systems were investigated in the neonatal Brazilian opossum, *Monodelphis domestica*. *Monodelphis* is a small pouchless marsupial whose young are born after 14 days of gestation in an extremely immature state while neurogenesis is still ongoing.

1) The developmental time course for synaptogenesis in the facial motor nucleus (FMN) and the hypoglossal motor nucleus (HMN) was examined using immunohistochemistry against: synaptic vesicle-associated proteins, synaptophysin and synaptotagmin; a synaptic membrane protein, SNAP-25; a growth cone protein, GAP-43; and microtubule-associated proteins, Tau-1 and MAP-2. We found that appearance of synapse-associated proteins is delayed in the FMN as compared to the HMN during the first two weeks of postnatal life.

2) To examine the postnatal development of the FMN and HMN we utilized retrograde tract tracing with cholera toxin subunit B (CtB). On the day of birth (1 PN), CtB labeled facial motoneurons were localized near the developing abducens nucleus. From 3 to 5 PN facial motoneurons were observed migrating to the FMN, and by 7 to 10 PN facial motoneurons had completed their migration. In contrast, CtB-labeled hypoglossal motoneurons were localized within the HMN from birth onward. Migrating facial motoneurons displayed a bipolar shape characteristic of migrating neurons, their rate of migration was faster than the rate of brainstem expansion. and they were localized in close proximity of vimentin immunostained radial glial fibers previously shown to guide migrating neurons.

3) Utilizing immunohistochemistry against choline acetyltransferase, neurofilament, and synaptotagmin we demonstrated that both facial and hypoglossal motoneuron projections extend to their respective target muscles and appear to innervate them from the day of birth. These results suggest that facial and hypoglossal motoneurons

innervate their target muscles at birth, during the period of facial motoneuron migration. Further, the FMN does not have synaptic or “classical” afferent innervation during this period. We suggest that the activity of facial motoneurons is regulated in a novel or distinct manner compared to hypoglossal motoneurons during this period of brain development.

CHAPTER ONE. GENERAL INTRODUCTION

In the class Mammalia, suckling is possibly the only behavior that is universal and also a characteristic of the class. Researchers have been interested in investigating this behavior because of its importance for the development and survival of mammalian young. Yet despite its importance in mammals, little is known about what factors at the level of the central nervous system regulate newborn suckling, or how these factors contribute to this behavior during growth and development in neonates.

Within the central nervous system, suckling behavior is thought to be regulated by motor cortex control of the oromotor system. The oromotor system is comprised of three motor nuclei of cranial nerves that control oral functions. The three motor systems are the trigeminal (5), facial (7), and hypoglossal (12) cranial nerves. The motoneuron component of the trigeminal cranial nerve innervates the muscles utilized in mastication (Carpenter, 1991; De Lahunta, 1983). Developing Brazilian opossum pups lack a developed, functional dentary-squamosal joint until after 14 PN (Filan, 1991; Müller, 1968). Since suckling behavior begins at birth, the trigeminal component probably is not utilized for this behavior during that period before joint formation. Projections from motoneurons within the hypoglossal motor nucleus innervate the intrinsic musculature of the tongue (Carpenter, 1991; De Lahunta, 1983). Innervation of the tongue musculature by the hypoglossal motoneurons plays a crucial role in suckling mechanisms and has been investigated in some detail (German and Crompton, 1996; Müller, 1968). Motoneuron projections from the facial motor nucleus innervate the muscles of facial expression, specifically the muscles of the ear, eyelids, nose, cheeks, lips, and the caudal digastricus (Carpenter, 1991; De Lahunta, 1983). In addition to the tongue muscles, we believe that suckling behavior might need to utilize musculature of the face which would be innervated by the facial motor system.

Development of cranial nerves, like the facial nerve, has been documented in Eutherian mammals (Ashwell and Watson, 1983; Auclair et al., 1996; Gasser and Hendrickx, 1967;

Pearson, 1946; Pearson, 1947; Sataloff, 1990). However, because most of this development is prenatal, these studies of cranial nerve development in placental mammals required the use of either *in utero* procedures (Ashwell and Watson, 1983; Auclair et al., 1996) or cesarean section and fixation at critical time points during development (Gasser and Hendrickx, 1967; Pearson, 1946; Pearson, 1947; Sataloff, 1990) for their descriptions of facial nerve ontogeny.

Eutherian, or placental mammals, complete most of their development within the uterus, joined to the mother via the placenta (Campbell, 1987). In contrast, marsupials are born very early and complete their development, usually in the pouch, fixed to the mother's teat while nursing. A notable difference exists between these two orders of mammals concerning the developmental stage of the neonate at the time of birth, in that the marsupial is born extremely early and continues to develop "ex utero." Marsupials are born in an extremely immature state that might be considered almost an embryonic condition when compared to the developmental stage of Eutherian young. Although, at birth developing marsupial neonates already possess functional systems for suckling and respiration. Thus, marsupials are a useful tool that present an attractive alternative to study neuroembryological events.

In these studies we have examined the development of several components of the developing facial and hypoglossal motor systems in the marsupial. These systems are thought to be involved in suckling behavior in these developing neonates. In addition, neurological development in marsupial neonates continues well after birth, as they have protracted neurogenesis continuing well into the postnatal period (Iqbal et al., 1995a; Jacobson, 1984; Kuehl-Kovarik et al., 1995b; Swanson et al., 1994).

To take advantage of the protracted, postnatal development that occurs in marsupials, our laboratory utilizes *Monodelphis domestica*, the Brazilian gray short-tailed opossum. *Monodelphis* is a small pouchless marsupial that breeds well in the laboratory (Fadem et al., 1982). The absence of a pouch makes the young very accessible to manipulations for *in vivo*, "ex utero" developmental studies, thereby circumventing technically difficult *in utero* surgical

procedures that are required for studies with more conventional laboratory animals such as rats and mice. At birth neonatal opossum pups are essentially capable of suckling behavior in addition to their homeostatic functions: breathing, circulation, digestion, etc. The Brazilian opossum presents an opportunity to examine suckling at a time when the development of motor systems might not yet be completed.

Our laboratory has previously described the ontogeny of several neurochemical systems in *Monodelphis* (Elmquist et al., 1992; Elmquist et al., 1993; Elmquist et al., 1994; Fox et al., 1991a; Fox et al., 1991b; Iqbal et al., 1995a; Iqbal et al., 1995b; Iqbal and Jacobson, 1995a; Iqbal and Jacobson, 1995b; Kuehl-Kovarik et al., 1995a; Kuehl-Kovarik and Jacobson, 1996; Kuehl-Kovarik et al., 1993a; Pearson et al., 1993). In addition, several other laboratories are utilizing *Monodelphis* to study development of central nervous system regions including: olfactory bulbs (Brunjes et al., 1992; Philpot et al., 1994), visual system (Guillery and Taylor, 1993; Rivkees et al., 1988; Sakaguchi et al., 1994; Stone et al., 1994; Taylor and Guillery, 1994; West-Greenlee et al., 1996), cerebral cortex (Saunders et al., 1989), hypothalamus (Rivkees et al., 1988; Schwanzel-Fukuda et al., 1988), brainstem (Wang et al., 1992), cerebellum (Dore et al., 1990), and spinal cord (Cassidy and Cabana, 1993; Mollgard et al., 1994; Nicholls et al., 1990; Stewart et al., 1991; Treherne et al., 1992).

Literature Review

Muscles that contribute to oromotor behaviors receive innervation essentially from three cranial nerve motor nuclei: trigeminal, facial, and hypoglossal (reviewed in Travers, 1995). The motoneurons from the trigeminal motor nucleus (TMN), control the muscles of mastication involved in jaw closing and jaw opening. Motoneurons located in the facial motor nucleus (FMN) innervate the muscles involved in facial expression and motoneurons located in the hypoglossal motor nucleus (HMN) innervate the muscles controlling movement of the tongue. This research will concentrate on the maturation of two oromotor nuclei that might participate in suckling behavior: the facial and hypoglossal motor nuclei.

The facial nerve is the seventh cranial nerve; it has motor, parasympathetic, and sensory components (Carpenter, 1991). Taste receptors on the anterior two-thirds of the tongue constitute the sensory component and innervation of the pterygopalatine and submandibular ganglions by the superior salivatory nucleus constitute the parasympathetic component. The motor component consists of motoneurons located in the facial motor nucleus (FMN) which innervate the muscles of facial expression and movement (supplying the ear, eyelid, nose, cheek, lips, and the caudal digastricus). The FMN is located in the ventral-lateral medulla; it is caudal to the trapezoid body, rostral to the inferior olivary nucleus, at the level of attachment of the caudal cerebellar peduncle to the cerebellum, and midway between the pyramid and the spinal tract of the trigeminal nerve (De Lahunta, 1983). Specific behaviors to which the FMN contributes would include suckling, attack, defense, emotion, and exploration.

The hypoglossal nerve is the twelfth cranial nerve; it has only a motor component (Carpenter, 1991). The musculature of the tongue is innervated by motoneurons located within the hypoglossal motor nucleus (HMN) that constitute the hypoglossal nerve. The HMN is located in the caudal-dorsal medulla, adjacent to the midline at the floor of the fourth ventricle (De Lahunta, 1983). The HMN would contribute to behaviors involved in suckling, licking, swallowing, and food intake in general.

The development of a functional motor system includes neurogenesis, neuronal migration, nucleus organization, cell death, and the establishment of both afferent and efferent synapses. This research is designed to study several of these phenomena during the maturation of the facial and hypoglossal motor nuclei in the Brazilian opossum.

Neurogenesis

Neurogenesis of the brainstem cranial motor nuclei appears to be a prenatal event in the Brazilian opossum (Jacobson laboratory, unpublished observations). The timing of the genesis of the facial and hypoglossal motoneurons is not known for the Brazilian opossum but has been investigated in detail in the neonatal rat (reviewed in Altman and Bayer, 1995a;

Altman and Bayer, 1995b). The rat FMN has a neurogenic gradient with the rostral neurons being “older” and the caudal neurons being “younger.” Specifically the rostral facial motoneurons are generated on embryonic days 12 (E12; 40%) and 13 (60%) while the caudal facial motoneurons are generated on E12 (10%), E13 (85%), and E14 (5%). Neurogenesis of the HMN occurs on E11 (10%) and E12 (90%). The HMN does not have a rostral to caudal gradient as observed in the FMN. Thus, the hypoglossal motoneurons are formed slightly earlier than the facial motoneurons.

Migration

Migration of facial motoneurons is also well characterized in the embryonic rat (Altman and Bayer, 1982; Altman and Bayer, 1995a; Altman and Bayer, 1995b). Following neurogenesis, from the midline neuroepithelium, the growth of efferent axons precedes the perikaryal migration through the brainstem. Perikaryal migration of facial motoneurons takes an initial course directed at a right angle away from their efferent axons. This initial right angle of migration helps to form the genu of the facial nerve with the efferent axons “looping” around the abducens cranial nucleus. Facial motoneurons migrate away from the dorsal midline of the medulla in a ventral-lateral direction toward the location of the “adult” FMN in the ventral-lateral region of the medulla. This complex pattern of migration is unique; it differs dramatically from and is best contrasted to trigeminal motoneurons migrating to their motor nucleus. Following neurogenesis, from the midline neuroepithelium, trigeminal motoneurons undergo perikaryal migration in a lateral direction to their motor nucleus first and then the growth of efferent axons follows. Hypoglossal motoneurons have a limited migration after neurogenesis from the neuroepithelium along the floor fourth ventricle to the adjacent location of the HMN.

Nucleus Organization

Previously researchers have reported that the FMN consists of several distinct subnuclei visible at the light microscopic level in the adult cat (Courville, 1966; Papez, 1927),

dog (Papez, 1927; Vraa-Jensen, 1942), guinea pig (Papez, 1927), monkey (Welt and Abbs, 1990), mouse (Ashwell, 1982), opossum [Brush-tailed (Provis, 1977), and North American (Dom, 1982)] and rat (Hinrichsen and Watson, 1984; Komiyama et al., 1984; Kume et al., 1978; Martin and Lodge, 1977; Papez, 1927; Semba and Egger, 1986; Watson et al., 1982). The number and shape of these subnuclei vary from species to species; however, the FMN can generally be subdivided into 5-6 subnuclei identified as medial, intermediate, lateral, dorsal lateral, and suprafacial (accessory facial). However, the FMN of the Brazilian opossum does not appear to contain separate subnuclei as observed in the other species. Also, the HMN of the Brazilian opossum and other species is not composed of distinct subnuclei as observed in the FMN.

Cell Death

The occurrence of cell death has been previously examined in the developing FMN of the mouse (Ashwell and Watson, 1983). In this study Ashwell and Watson examined cell death through utilization of motoneuron counts at different time points. Their study concluded that of the number of motoneurons present on embryonic day 17 (E17) in the FMN 68% disappear before adulthood with the highest losses occurring between E18 to E20. Motoneurons with pyknotic nuclei were observed on E17 and were most numerous on E18 leading to the conclusion that the loss of motoneurons was mainly a result of cell death. Utilizing retrograde tract tracing they reported that facial motoneurons were connected to their respective target muscles before the majority of cell death occurs.

Efferent Innervation

Previous studies, utilizing retrograde tract tracing, have described the adult innervation of peripheral musculature in multiple species (Ashwell and Watson, 1983; Kume et al., 1978; Populin and Yin, 1995; Provis, 1977; Senba et al., 1987; Watson et al., 1982; Welt and Abbs, 1990). In addition, these studies indicate that the innervation pattern forms a topographically ordered map within the FMN of the adult cat (Courville, 1966; Papez, 1927), dog (Papez,

1927; Vraa-Jensen, 1942), guinea pig (Papez, 1927), monkey (Welt and Abbs, 1990), mouse (Ashwell, 1982), opossum [Brush-tailed (Provis, 1977), and North American (Dom, 1982)] and rat (Hinrichsen and Watson, 1984; Komiyama et al., 1984; Kume et al., 1978; Martin and Lodge, 1977; Papez, 1927; Semba and Egger, 1986; Watson et al., 1982). Throughout the studies there is general agreement that the caudal (auricular) muscles are innervated by motoneurons in the medial aspects of the FMN; dorsal (ocular) muscles are innervated by motoneurons in the dorsal aspects of the FMN; ventral muscles are innervated by motoneurons in the ventral aspects of the FMN; and the rostral (nasolabial) muscles are innervated by motoneurons in the lateral aspects of the FMN.

In addition, previous reports using retrograde tract tracing of facial motoneurons in the mouse suggest that facial motoneurons are connected to their target muscles by embryonic day 17 (Ashwell and Watson, 1983). In the neonatal rat, studies indicate that the facial motoneurons innervate their respective target muscles by birth [1 PN, personal observations; (Klein et al., 1990)] or soon after [5 PN, (Senba et al., 1987; Yokosuka and Hayashi, 1992)]. The study by Klein and colleagues (1990) reported that the innervation pattern of facial musculature from the FMN was similar when compared to adult rats. However, the expected time course of innervation for facial motoneurons in the rat would be embryonic, similar to the mouse, and has not been examined.

Previous studies have examined the innervation of the hypoglossal motoneurons in 6 day old postnatal rat pups (Senba et al., 1987) and in the adult (Aldes, 1995; Barnard, 1940; Krammer et al., 1979; Lewis et al., 1971). The somatotopic organization of the HMN has been investigated by retrograde degeneration (Barnard, 1940), tract tracing (Aldes, 1995; Krammer et al., 1979), and cholinesterase histochemistry (Lewis et al., 1971). The studies are in general agreement that the dorsal aspects of the HMN innervate tongue retrusor muscles and the ventral aspects of the HMN innervate the tongue protrusor muscles.

Afferent Innervation

A number of studies describe the afferent projections to the motor nuclei that play a role in ingestive behaviors. Afferent projections to the trigeminal, facial, and hypoglossal motor nuclei from the reticular formation are involved in oral-facial functions in the adult rat (reviewed in Travers, 1995; Travers and Norgren, 1983). The rat FMN receives afferent innervation from nuclei within the midbrain, pons, and medulla. In fact, most of the projections to oromotor nuclei are from neurons within the reticular formation. Neurons from within the reticular formation that project to the TMN are located rostral to neurons projecting to the HMN, which are rostral to neurons projecting to the FMN. The FMN also receives afferent input from other sources. Projections to the FMN from within the midbrain are from central gray, lateral lemniscus, midbrain reticular formation, olivary pretectal nucleus, periocular nuclei, red nucleus, and superior colliculus (Bystrzycka and Nail, 1983; Travers, 1995; Travers and Norgren, 1983). Pontine projections to the FMN are from the Kölliker-Fuse nucleus, ventrolateral parabrachial nucleus, sensory trigeminal nucleus, and pontine reticular formation. Medullary projections to the FMN are from the caudal spinal trigeminal complex, medial vestibular nuclei, nucleus of the solitary tract, and medullary reticular formation. Borke and coworkers (1983) reported, in the adult rat, that the HMN receives its primary projections from the reticular formation, nucleus of the solitary tract, and spinal trigeminal nucleus, with additional projections from several other nuclei similar to the FMN.

The peptidergic and aminergic innervation of the FMN in the neonatal rat shows an increase during the first week of postnatal life, with an established innervation pattern appearing by the tenth postnatal day (Senba et al., 1985). The FMN receives peptidergic and aminergic innervation in the way of acetylcholine from the reticular formation, met-enkephalin from the olivary pretectal nucleus and reticular formation, noradrenaline from the Kölliker-Fuse and subceruleus nucleus, serotonin from the raphe nuclei, and substance P from the periocular nuclei and reticular formation (reviewed in Senba et al., 1985; Travers, 1995).

In the adult North American opossum (*Didelphis virginiana*), mesencephalic projections to oral motor nuclei strongly favor the FMN (Panneton and Martin, 1978; Panneton and Martin, 1979; Panneton and Martin, 1983). In addition, the opossum FMN receives afferent innervation from brainstem areas such as the reticular formation, nucleus of the solitary tract, and trigeminal sensory nucleus (reviewed in Dom et al., 1973; Panneton and Martin, 1983), similar to the rat (Travers, 1995; Travers and Norgren, 1983).

Transient Receptors

In the neonatal rat, facial motoneurons have been shown to transiently express a number of compounds and receptors. Tribollet and coworkers (1991) have reported a transient expression of vasopressin binding sites between E17 and 19 PN in the FMN of the rat. In extracellular recordings from facial motoneurons, the application of arginine vasopressin resulted in neuronal excitation (acting through a V₁ type receptor) indicating formation of functional postsynaptic receptors and second messenger systems. In addition, the HMN expresses vasopressin binding sites from 1 to 25 PN in the neonatal rat and lower levels in the adult (Tribollet et al., 1991). Yokosuka and Hayashi (1992) have described estrogen-receptor-like-immunoreactivity being transiently expressed in the FMN of rat pups from postnatal day 1 through 11. Both facial and hypoglossal motoneurons transiently express nerve growth factor (NGF) receptors during development (Friedman et al., 1991; Yan and Johnson, 1988). In the developing rat, these NGF receptors are expressed from E15 to 10 PN by facial and hypoglossal motoneurons.

Both the facial and hypoglossal motoneurons transiently express adenosine deaminase (ADA) in the rat during development (Senba et al., 1987). Facial motoneurons express ADA from E18 to 15 PN while hypoglossal motoneurons express ADA from E15 to 25 PN. The significance of the transient expression of ADA is not known, but it is suggested that the metabolic pathway associated with ADA is important in the maturation of these motoneurons.

Our laboratory has reported a transient expression of cholecystikinin (CCK) binding

sites in the FMN of the Brazilian opossum (Kuehl-Kovarik et al., 1993b) and neonatal rat (Kuehl-Kovarik and Jacobson, 1996). In the Brazilian opossum, CCK binding sites are present in the developing hindbrain from birth and can be localized to the FMN by 10 PN. They are abundant through 35 PN, then decrease at 45 PN and disappear by 60 PN, then return in the adult. In addition, our laboratory demonstrated that CCK binding sites are transiently expressed in the FMN of the laboratory rat from E20 through 10 PN (Kuehl-Kovarik and Jacobson, 1996). In the Brazilian opossum, binding sites for CCK are expressed diffusely in the region of the HMN from birth until 25 PN, when they are discretely localized in the HMN. In both the opossum and rat, CCK binding sites are present in the FMN in neonatal pups and disappear prior to weaning. The significance of these binding sites in relation to the development of the FMN or neonatal behavior is unknown.

Dissertation Organization

The body of this dissertation consists of three manuscripts. The first manuscript has been published in the *Journal of Comparative Neurology* [368: 270-284, (1996)]. The second and third manuscripts will be submitted to the journal *Developmental Brain Research*. The papers are preceded by a general introduction and followed by a summary and general discussion. The literature cited in the introduction and discussion is listed after the general discussion section. All of the research presented in this dissertation was performed by myself, under the guidance of Dr. Carol D. Jacobson and Dr. M. Cathleen Kuehl-Kovarik.

CHAPTER TWO. CHARACTERIZATION AND ONTOGENY OF SYNAPSE-ASSOCIATED PROTEINS IN THE DEVELOPING FACIAL AND HYPOGLOSSAL MOTOR NUCLEI OF THE BRAZILIAN OPOSSUM BRAIN

A paper published in the Journal of Comparative Neurology

Jack J. Swanson, M. Cathleen Kuehl-Kovarik, Michael C. Wilson,
Joel K. Elmquist, and Carol D. Jacobson

Abstract

The characterization and ontogeny of synapse-associated proteins in the developing facial and hypoglossal motor nuclei were examined in the Brazilian opossum (*Monodelphis domestica*). Immunohistochemical markers utilized in this study were: synaptic vesicle-associated proteins, synaptophysin and synaptotagmin; a synaptic membrane protein, plasma membrane-associated protein of 25 kDa (SNAP-25); a growth cone protein, growth-associated phosphoprotein-43 (GAP-43); and microtubule-associated proteins, axonal marker Tau and dendritic marker microtubule-associated protein-2 (MAP-2). In this study we have found that during the first ten postnatal days (1-10 PN), the facial motor nucleus lacked immunoreactivity for synaptophysin, synaptotagmin, GAP-43, Tau, and SNAP-25. After 10 PN, immunoreactivity increased in the facial motor nucleus for synaptophysin, synaptotagmin, GAP-43, and Tau, whereas immunoreactivity for SNAP-25 was not evident until between 15 and 25 PN. Conversely, immunoreactivity for MAP-2, was present in the facial motor nucleus from the day of birth. In contrast, the hypoglossal motor nucleus displayed immunoreactivity from 1 PN for synaptophysin, synaptotagmin, SNAP-25, GAP-43, Tau, and MAP-2. These

results suggest that the facial motor nucleus of the opossum may not receive afferent innervation as defined by classical synaptic markers until 15 PN, and further, that characteristic mature synapses are not present until between 15 and 25 PN. These results indicate that there may be a delay in synaptogenesis in the facial motor nucleus as compared to synaptogenetic events in the hypoglossal motor nucleus. Since the facial motor nucleus is active prior to completion of synaptogenesis we suggest that the facial motoneurons are regulated in a novel or distinct manner at this time period.

Introduction

Neuron proliferation, migration, process extension, and synaptogenesis make up a complex procession of events that is precisely timed and regulated during development of the central nervous system (CNS). The characterization of the temporal expression of markers of synaptogenesis during neurogenesis and development will further our understanding of the complexity of the developing mammalian CNS. This study utilizes markers of process extension and synaptogenesis in order to compare the time course of the afferent innervation to the facial and hypoglossal motor nuclei during development.

In general, the identification of the time course of developmental events for cranial motoneurons often requires the use of *in utero* procedures. The use of marsupials offers an attractive alternative for *in vivo* studies of the developing CNS. Our laboratory has previously described the ontogeny of several neurochemical systems in the Brazilian short-tailed opossum, *Monodelphis domestica* (Fox et al., 1991a,b; Elmquist et al., 1992, 1993, 1994; Kuehl-Kovarik et al., 1993a). *Monodelphis* is a small pouchless marsupial that breeds well in the laboratory. The young are born in an extremely immature state with a protracted postnatal period of neurogenesis (Jacobson, 1984; Iqbal et al., 1995; Swanson et al., 1994). The absence of a pouch makes the young very accessible to manipulations for *in vivo*, "ex utero" developmental studies.

Our recent studies have focused upon the distribution of neuropeptides and their

receptors during development. Facial motoneurons transiently express cholecystinin (CCK) binding sites between day 1 of postnatal life (1 PN) and 35 PN, disappearing by 60 PN in the Brazilian opossum (Kuehl-Kovarik et al., 1993a) We have also shown that CCK binding sites are transiently expressed in the facial motor nucleus of the laboratory rat (Kuehl-Kovarik et al., 1993b). Along with the facial motor nucleus, the hypoglossal motor nucleus is involved in food intake and swallowing. However, in the Brazilian opossum, CCK binding sites become diffusely evident in the area of the hypoglossal motor nucleus at 10 PN and are subsequently consistently expressed from 25 PN (Kuehl-Kovarik et al., 1993a). These results indicate possible differential regulation of the two motor nuclei during the early postnatal period.

One method of investigating potential differential regulation of the oromotor nuclei involves determining the time of synaptogenesis for each of the motor nuclei. Previously, others have mapped the development of brain regions using several different immunohistochemical markers of synaptogenesis. For example, synaptic formation has been reported to correlate with the expression of synaptophysin (p38; Masliah et al., 1991; Laemle et al., 1991; Leclerc et al., 1989), synaptotagmin (p65; Lou and Bixby, 1993), synaptosomal-associated protein of 25 kDa (SNAP-25; Bark, 1993; Catsicas et al., 1991), growth-associated phosphoprotein-43 (GAP-43; Dani et al., 1991), synapsin I (Moore and Bernstein, 1989), and synaptophorin (synaptophysin II; Lou and Bixby, 1993).

These proteins are found in various components of the neuron. Synaptophysin is a major integral protein of synaptic vesicles and its presence is required for the fusion of the vesicle preceding neurotransmitter release (Greengard et al., 1993). The developmental profile of synapses has been shown to correlate with the distribution of synaptophysin immunoreactivity as seen with the light microscope (Voigt et al., 1993). Synaptotagmin is also localized in synaptic vesicles (Südhof and Jahn, 1991; Walch-Solimena et al., 1993) and is needed for the secretory process. Synaptotagmin is believed to play a role in either the docking of the vesicle at the release site or in the fusion of the vesicle with the plasma membrane

(DeBello et al., 1993; Greengard et al., 1993; O'Connor et al., 1994). Utilization of SNAP-25 immunohistochemistry may provide insight into the formation of mature synapses. The expression of SNAP-25 (a protein located on the presynaptic plasma membrane) mRNA and protein have been found to be correlated with the time of synaptogenesis (Catsicas et al., 1991). SNAP-25 is hypothesized to have an important role during vesicle docking and fusion as a 25 kDa SNAP receptor (SNARE) protein (Wilson and Bark, 1994). Immunocytochemical studies of SNAP-25 have reported a cellular localization with an accompanying shift from axons and cell bodies to the presynaptic terminals (Oyler et al., 1991). The shift of SNAP-25 protein during subcellular development suggests that SNAP-25 may play a role in the establishment and stabilization of specific presynaptic terminals in the brain. Immunoreactivity for GAP-43 has been used as a marker of axonal growth cones and can be used to distinguish axonal from dendritic growth cones (Goslin et al., 1988). Tau is a microtubule-associated protein (MAP) that has been localized in the axonal process while MAP-2 is another MAP that is found specifically in the soma and dendritic processes of neurons (Ferreira et al., 1987).

In order to look at the potential role of afferent input to the motor nuclei during development, the present study examines the characterization and ontogeny of the synapse-associated proteins in the facial and hypoglossal motor nuclei of the newborn Brazilian opossum brain. We have characterized the distribution in the facial motor nucleus and hypoglossal motor nucleus for: synaptophysin, synaptotagmin, SNAP-25, GAP-43, paired helical filaments-1 (TAU; PHF-1), and MAP-2 during postnatal development.

Materials and Methods

Animals

Developing Brazilian gray short-tailed opossums were obtained from a colony at Iowa State University. Animals used to start and maintain the breeding colony were obtained from the Southwest Foundation for Research and Education (San Antonio, TX). Opossums were individually housed in plastic cages, maintained at a constant temperature (26°C) with a 14:10

light-dark cycle, with food and water available ad libitum (Reproduction Fox Chow: Milk Specialties Products, Madison, WI). The animals and procedures used were in accordance with the guidelines and approval of the Iowa State University Committee on Animal Care. To obtain pups, male and female animals were paired for breeding for 14 days and then separated. Females were then checked daily at 1500 hours for the presence of pups (day of birth = day 1 of postnatal life; 1 PN). After a gestational period of 14 days (Fadem et al., 1982), pups are born in an extremely immature state, open their eyes around 30 PN, and are weaned at 60 PN (Schwanzel-Fukuda et al., 1988). At least three animals from a minimum of three different litters were used at each of the following time points (1, 3, 5, 7, 9, 11, 13, 15, and 25 PN) for the immunohistochemical study. Adult female opossums used for immunoblotting were obtained from the colony described above.

Adult female rats (Harlan Sprague-Dawley, Indianapolis, IN) used in this study were maintained at Iowa State University. Rats were housed in plastic cages, maintained at a constant temperature (23°C) with a 14:10 light-dark cycle, with food and water available ad libitum (Teklad Rodent Diet: Harlan Sprague-Dawley).

Tissue and Protein Preparation

Immunoblotting: In order to determine the ability of using these antibodies to characterize immunohistochemical patterns in the opossum we have chosen to immunoblot the opossum brain with three of the antisera. Thus, brain protein samples from adult female rats and adult female opossums were utilized in this portion of the studies. Following decapitation, brains were removed from the calvaria, blocked in the coronal plane at the level of the mammillary body into forebrain and hindbrain samples, and frozen in liquid nitrogen. Brains were homogenized in sample buffer (50 mM Tris-HCl, 10% glycerol, 0.05% SDS, pH 6.9) and protein was extracted as described previously (Sakaguchi et al., 1989; Elmquist et al., 1994).

Immunohistochemistry: Brains were collected from 1, 3, 5, 7, 9, 11, 13, and 15 PN opossums. Animals were anesthetized by cooling in a -15°C freezer and then decapitated. Heads were placed in Zamboni's fixative for 48 hours at 23°C. After fixation, the heads were infiltrated with 30% sucrose for 24 hours at 4°C. Brains were cut into 20 µm thick coronal sections on a cryostat (Reichert-Jung 2800N). Sections were thaw mounted onto poly-L-lysine (Sigma) coated slides and stored at 4°C until processed for immunohistochemistry.

Twenty-five day old opossums were anesthetized by cooling. After cooling, animals were perfused transcardially with 15 ml of Zamboni's fixative. The heads were isolated and postfixed in Zamboni's fixative for 48 hours. After fixation the brains were processed as described above.

Antiserum

Antibodies used in this study have been previously characterized in the rat. Table 2.1 lists the antibody dilution used for immunohistochemistry and immunoblotting, source, antibody host, and appropriate references.

Immunoblotting

Characterization of antigens was performed using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting procedures. The protocol utilized for immunoblotting was a modification of that reported previously for the developing opossum brain (Elmqvist et al., 1994). Brain protein homogenates were boiled in SDS reducing buffer (0.5M Tris-HCl, 10% SDS, 10% glycerol, 5% β-mercaptoethanol, and 2.5% bromophenol blue) for 4 minutes followed immediately by centrifugation. Each protein sample (20 µl) was loaded into respective lanes of a four percent stacking gel and separated on a ten (p65), twelve (p38), or fifteen (SNAP-25) percent separating gel using a vertical Mini-Protean II system (BIORAD). Gel electrophoresis was carried out under conditions described by Laemmli (1970). Following electrophoresis, proteins were transferred onto a polyvinylidene difluoride (PVDF; BIORAD) membrane in transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20% v/v

Table 2.1. Antisera utilized for Immunohistochemistry and Immunoblotting

Name	Immunohistochemistry	Immunoblotting	Source	Antibody Host	Reference
GAP-43	1:2,000	NA	Boehringer	Mouse	Goslin et al., 1988
			Mannheim		Goslin et al, 1990
MAP-2	1:1,000	NA	Boehringer	Mouse	Binder et al., 1986
			Mannheim		Caceres et al., 1986
PHF-1 (Tau-1)	1:30	NA	S Greenberg	Mouse	Greenberg & Davies, 1990 Greenberg et al., 1992
SNAP-25	1:2,000	1:1,000	M. C. Wilson	Rabbit	Catsicas et al., 1991 Oyler et al., 1991
Synaptophysin (p38)	1:2,000	1:1,000	A. J. Czernik	Mouse	Jahn et al., 1985
Synaptotagmin (p65)	1:1,000	1:1,000	R. Jahn	Mouse	Perin et al., 1990 Brose et al., 1992

methanol, pH 8.3). Following transfer, membranes were incubated in blocking buffer (1% BSA and 0.05% Tween-20 in Tris-buffered saline; TBST) for 2 hours at room temperature with gentle agitation. The blots were incubated overnight at 4°C in one of the primary antisera described (see Table 2.1). After washing in TBST, blots were then exposed to a biotinylated secondary antibody (Vector; 1:3000) generated against the host of the primary antibody (either goat anti-rabbit or horse anti-mouse) for one hour at room temperature. After washing in TBST, amplification of the signal was achieved by using a solution of streptavidin (Vector; 2 mg/ml, 1:3000) and biotinylated alkaline phosphatase (Vector; 1:3000) at room temperature for one hour. Immunoreactive bands were visualized with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) / Nitroblue tetrazolium (NBT) alkaline phosphatase color development reagents (BIORAD) for 5-10 minutes at room temperature. Apparent molecular weights were estimated by comparison with biotinylated molecular weight standards (BIORAD) run concurrently on each gel.

Immunohistochemistry

The protocol utilized for immunohistochemistry was a modification of that previously reported (Fox et al., 1991a; Elmquist et al., 1992, 1994) by our laboratory for the Brazilian opossum brain. The slide mounted sections were rinsed, incubated with a H₂O₂ solution to remove endogenous peroxidase activity, exposed to normal serum as a blocking agent and then incubated in primary antibody for 20 hours at room temperature (see Table 2.1 for dilution used). After adequate washing, the tissue sections were incubated in either horse anti-mouse (Vector; 1:600) or goat anti-rabbit IgG (Vector; 1:600) (depending upon the species that the primary antisera was generated in) for 2 hours at room temperature, rinsed, and reacted with avidin-biotin complex (Vector Elite Kit; 1:200) at room temperature for an additional hour. After washing, the tissue sections were stained by exposing them to a substrate composed of a 3,3' diaminobenzidine tetrahydrochloride (DAB; Sigma), nickel sulfate (Fisher Scientific), and H₂O₂, all dissolved in 0.1 M sodium acetate. After staining for 10-18 minutes, the reaction

was terminated by rinsing the slides in two successive rinses of 0.9% saline. Sections were counterstained with 0.5-1.0% neutral red (Fisher Scientific) and then dehydrated in graded alcohols, cleared in xylene and coverslipped with permount mounting media (Fisher Scientific) and analyzed and photographed with a light microscope (Axiophot, Carl Zeiss). Each run contained negative controls generated by the omission of the primary antiserum. Omission of the primary antisera resulted in no specific staining at any of the ages examined.

Analysis of Immunohistochemical Tissue

Sections were examined with a Zeiss Axiophot microscope and regions containing immunoreactivity were identified and recorded on maps of coronal sections of the opossum brain (Fox et al., 1991a,b; Elmquist et al., 1992, 1994) as needed. Structures were identified by reference to an atlas of the developing rat brain (Paxinos et al., 1991; Paxinos et al., 1994; Altman and Bayer, 1995c).

Digital Processed Figures

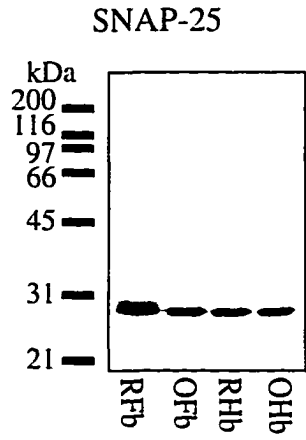
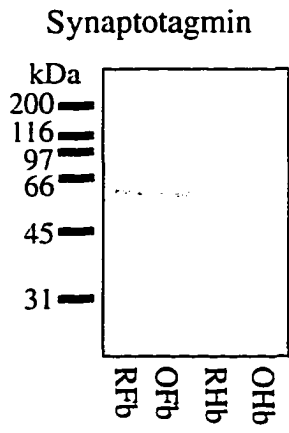
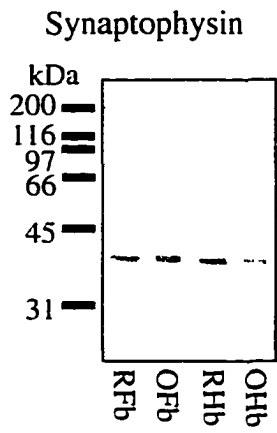
Figure 1 was produced digitally on a Macintosh 660AV computer. To create this figure, immunoblots were digitized with a flatbed scanner (Hewlett Packard Scanjet II) and transferred to Adobe Photoshop v2.5. Images were then cropped to size and saved as tiff files for labeling. Aldus Freehand v3.0 was utilized for labeling the figure and the final image was printed on an Imagesetter at 2400 dots per inch by 150 lines per inch.

Results

Immunoblot Analysis

Antisera generated against synaptophysin (Fig. 2.1A), synaptotagmin (Fig. 2.1B), and SNAP-25 (Fig. 2.1C) demonstrated generally good cross reactivity between the rat and the Brazilian opossum. These antibodies each detected a major band with the apparent molecular weights for each synaptophysin, synaptotagmin, and SNAP-25 (Fig. 2.1) as has been shown in the rat. Previously, a molecular weight of 38 kDa was reported for synaptophysin (Jahn et al., 1985), 65 kDa for synaptotagmin (Matthew et al., 1981), and 25 kDa for

Figure 2.1. Representative immunoblots demonstrating cross reactivity for (A) synaptophysin-like immunoreactivity, (B) synaptotagmin-like immunoreactivity, and (C) synaptosomal-associated protein 25 (SNAP-25)-like immunoreactivity. Antibodies detected bands with molecular weights similar to molecular weights obtained from previous reports. Molecular weight markers are indicated at the right of each blot. Labels under each lane indicate the species and region from which the protein samples were obtained. Abbreviations: RFb, Rat forebrain; OFb, Opossum forebrain; RHb, rat hindbrain; and OHb, Opossum hindbrain.



SNAP-25 (Oyler et al., 1991).

Immunohistochemical Analysis

Distribution of Synaptic Vesicle-Associated Proteins: Synaptophysin and Synaptotagmin

At the time of birth the brain of *Monodelphis* is extremely immature, and grows and matures rapidly during the first month of postnatal life. Synaptophysin-like immunoreactivity and synaptotagmin-like immunoreactivity were both seen in the postnatal brainstem at all ages examined (Figs. 2.2 and 2.3). In the facial motor nucleus, synaptophysin-like immunoreactivity and synaptotagmin-like immunoreactivity were both absent from the facial motor nucleus until 9 PN (Figs. 2.2A and 2.3A). The immunoreactivity gradually increases in the nucleus until 15 PN when the nucleus contains the full complement of immunoreactive elements (Figs. 2.2C and 2.3C). In general, immunoreactive elements for both synaptophysin and synaptotagmin appear in the facial motor nucleus initially at the border of the facial motor nucleus with increasing immunoreactivity towards the central region of the nucleus (Fig. 2.4). In addition, with increasing age the immunoreactive elements appear in the medial region before appearing in the lateral aspects (Figs. 2.4B-E). Synaptophysin-like immunoreactivity and synaptotagmin-like immunoreactivity both appeared to have a similar distribution (Figs. 2.2 B,D and 2.3 B,D) in the facial motor nucleus. Specifically, synaptophysin-like immunoreactivity and synaptotagmin-like immunoreactivity were always absent from the facial motoneuron cell bodies and were distributed within the neuropil of the facial motor nucleus by 15 PN (Figs. 2.2 C,D and 2.3 C,D).

In the hypoglossal motor nucleus immunoreactivity for synaptophysin and synaptotagmin were observed at all ages examined (Figs. 2.2E-H and 2.3E-H). The immunoreactivity present in the hypoglossal motor nucleus did not change during the first fifteen days postnatally (Figs. 2.2E,G and 2.3E,G) as seen for the facial motor nucleus. As seen for the facial motor nucleus, synaptophysin-like immunoreactivity and synaptotagmin-like immunoreactivity displayed a similar distribution (Figs. 2.2F,H and 2.3F,H), which was

Figure 2.2. Immunoreactivity for synaptophysin is absent until 9 days postnatal (PN) and becomes apparent in the facial motor nucleus by 15 PN. Photomicrographs demonstrate synaptophysin-like immunoreactivity in the developing facial motor nucleus at 5 PN at a low (A) and higher (B) magnification and at 15 PN at a low (C) and higher (D) magnification. In contrast, immunoreactivity for synaptophysin is found in the developing hypoglossal motor nucleus at all ages examined. Photomicrographs display synaptophysin-like immunoreactivity in the developing hypoglossal motor nuclei at 5 PN at low (E) and higher (F) magnifications and at 15 PN at low (G) and higher (H) magnifications. Synaptophysin-like immunoreactivity is found in the neuropil surrounding unstained cell bodies (asterisks). High magnification photomicrographs are taken from the left nuclei of interest (box). Note that the cartilage of the developing skull is dark due to counterstaining of the tissue and does not represent specific immunoreactivity. Scale bars = 150 μm in A, C, E, and G, and 15 μm in B, D, F, and H.

Synaptophysin

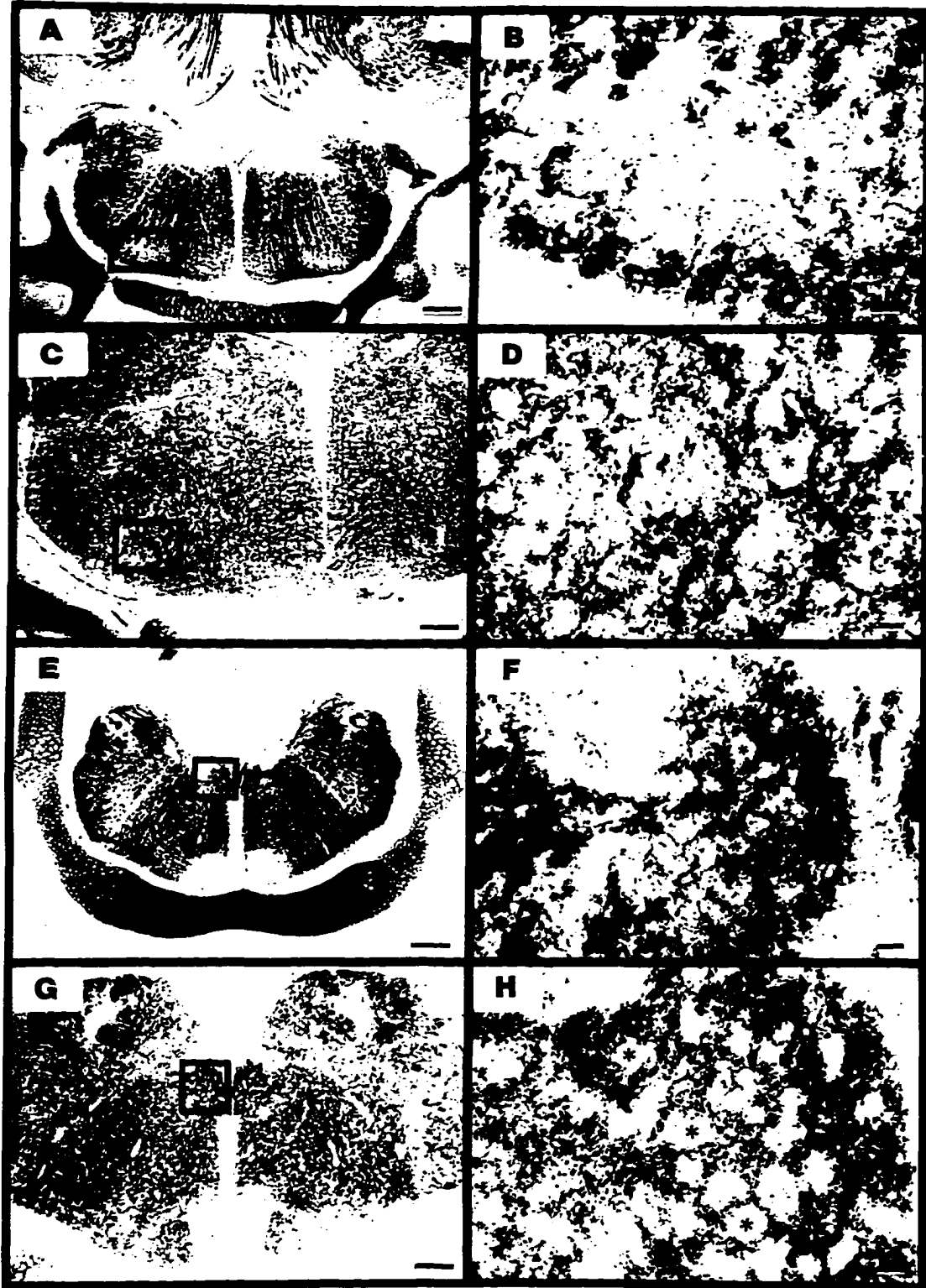


Figure 2.3. Immunoreactivity for synaptotagmin is absent until 9 days postnatal (PN) and becomes apparent in the facial motor nucleus by 15 PN following a similar time course of expression as found for synaptophysin. Synaptophysin-like immunoreactivity is demonstrated in the photomicrographs for the developing facial motor nucleus at 5 PN at low (A) and higher (B) magnifications and at 15 PN at low (C) and higher (D) magnifications. Immunoreactivity for synaptotagmin is found in the developing hypoglossal motor nucleus at all ages examined similar to that seen for synaptophysin. Photomicrographs display synaptotagmin-like immunoreactivity in the developing hypoglossal motor nuclei at 5 PN at low (E) and higher (F) magnifications and at 15 PN at low (G) and higher (H) magnifications. Synaptotagmin-like immunoreactivity in the neuropil surrounds unstained cell bodies (asterisks). High magnification photomicrographs are taken of the left nuclei of interest (box). Note that the cartilage of the developing skull is dark due to counterstaining of the tissue and does not represent specific immunoreactivity. Scale bars = 150 μm in A, C, E, and G, and 15 μm in B, D, F, and H.

Synaptotagmin

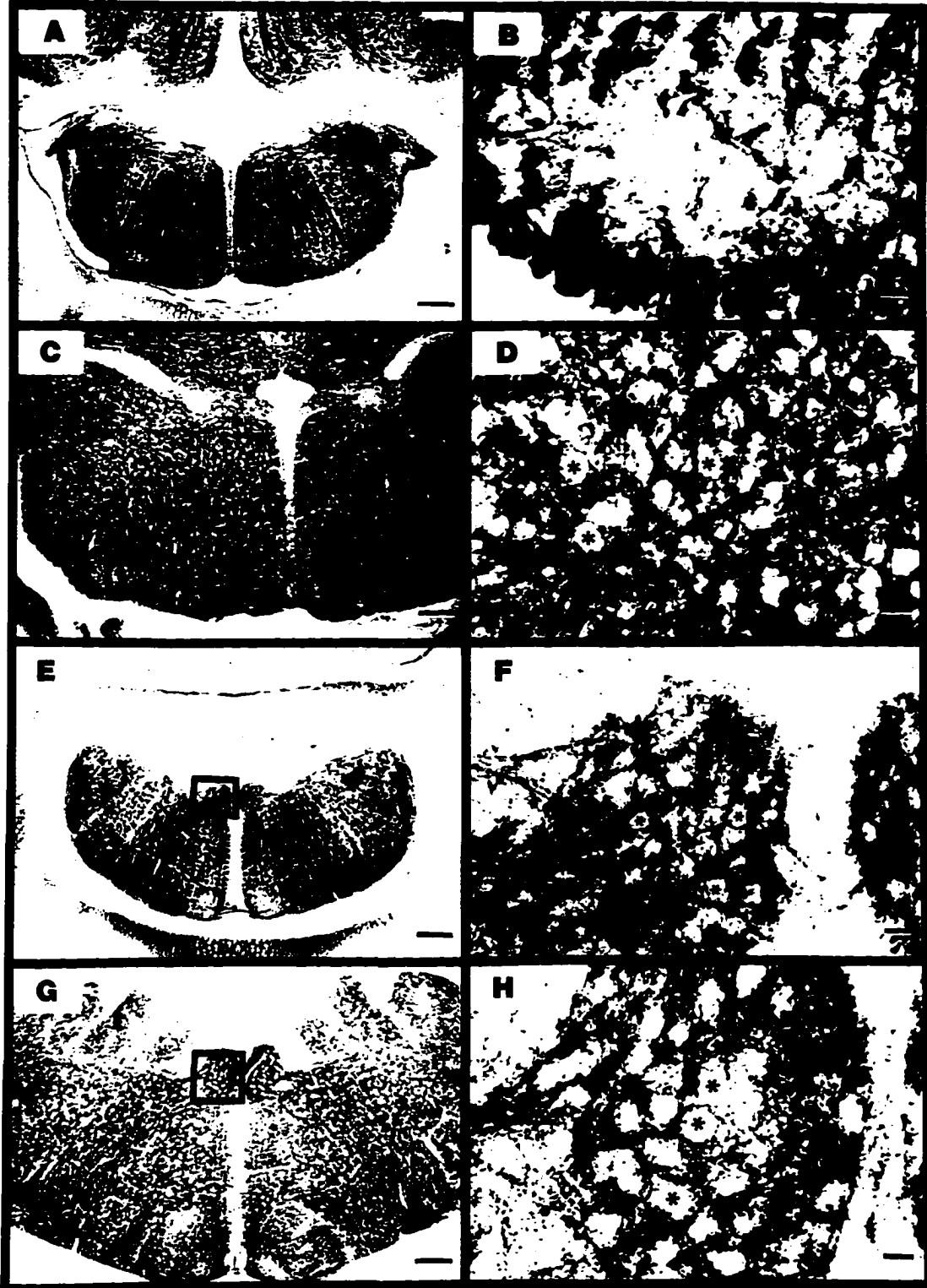
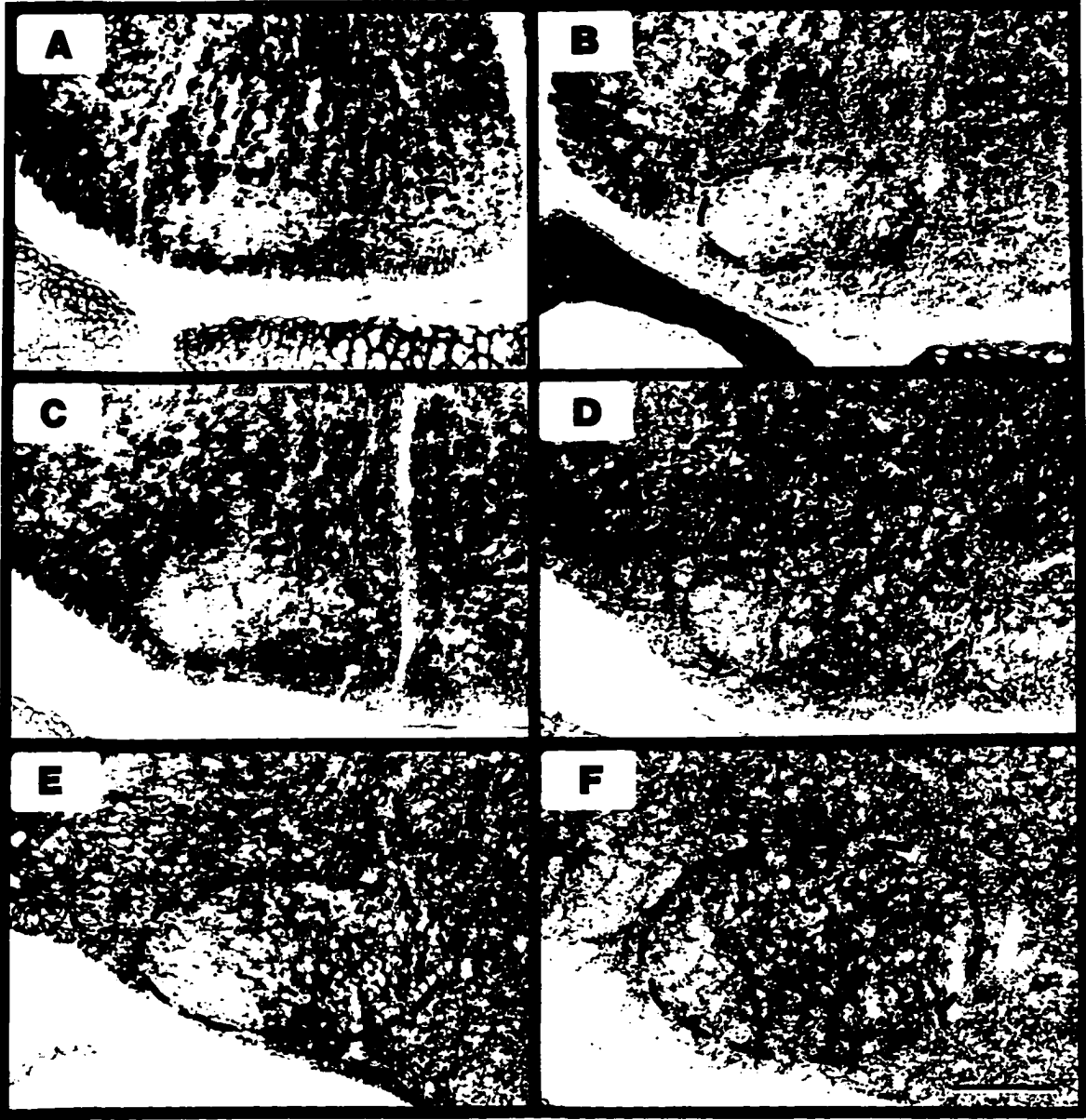


Figure 2.4. Photomicrographs demonstrating synaptophysin-like immunoreactivity in the developing facial motor nucleus. Immunoreactive elements initially appear around the border of the facial motor nucleus. With increasing age there is an increase in immunoreactivity towards the central region filling in the nucleus by 15 days postnatal (PN) (**F**). In addition, the immunoreactive elements appear in the medial region before appearing in the lateral aspects, 5 PN (**A**), 7 PN (**B**), 9 PN (**C**), 11 PN (**D**), and 13 PN (**E**). Scale bar = 200 μm .



always absent from the cell bodies, and was restricted to the neuropil of the hypoglossal motor nucleus.

Distribution of Markers of Axonal Growth: SNAP-25 and GAP-43

Synaptosomal-associated protein 25-like immunoreactivity (SNAP-25-IR) demonstrated a pattern of expression similar to that for the synaptic vesicle-associated proteins. However, the time course of expression in the facial motor nucleus for SNAP-25 was later than that for the synaptic vesicle-associated proteins. SNAP-25-IR was absent from the facial motor nucleus until 15 PN (Fig. 2.5A). The SNAP-25-IR increased in the facial motor nucleus between days 15 PN through 25 PN (Fig. 2.5C). In general, the immunoreactivity for SNAP-25 appeared first in the borders of the facial motor nucleus. With increasing age there was a gradual increase in immunoreactivity from the peripheral to the central regions of the facial motor nucleus. In addition to the increasing area of immunoreactive elements they appear in the medial region before in the lateral region.

The hypoglossal motor nucleus contained SNAP-25-IR from day 1 PN (Figs. 2.5E,G). From 1-5 PN immunoreactivity for SNAP-25 was dispersed, however, immunoreactive elements became more common in the hypoglossal motor nucleus after 5 PN (Figs. 2.5E-H).

Immunoreactivity for GAP-43 (GAP-43-IR) resembled that for the synaptic vesicle-associated proteins synaptophysin and synaptotagmin in their time course of expression. The GAP-43-IR was seen at all ages studied beginning at 1 PN in the developing brainstem. However, the region of the developing facial motor nucleus is virtually absent of GAP-43-IR until 11 PN (Fig. 2.6A,B). From 11 through 15 PN (Fig. 2.6C,D) the facial motor nucleus becomes infiltrated with immunoreactivity. Immunoreactive elements for GAP-43 appear in the facial motor nucleus in the same pattern as observed for synaptophysin, synaptotagmin, and SNAP-25 with immunoreactivity appearing first at the borders of the nucleus and also in a medial to lateral increase over time. In addition, the GAP-43-IR appears to be restricted to the

Figure 2.5. Photomicrographs demonstrating synaptosomal-associated protein 25-like immunoreactivity (SNAP-25-IR) in the developing facial motor nucleus. The developing facial motor nucleus lacks SNAP-25-IR until 14 days postnatal (PN). Immunoreactivity is found throughout the nucleus at 25 PN. Immunoreactivity for SNAP-25 is displayed at 5 PN at low (A) and higher (B) magnifications and at 25 PN at low (C) and higher (D) magnifications. The developing hypoglossal motor nucleus displays SNAP-25-IR consistently from the day of birth. Photomicrographs show SNAP-25-IR in a 5 PN opossum at low (E) and higher (F) magnifications and a 15 PN opossum at low (G) and higher (H) magnifications. Immunoreactivity for SNAP-25 is found in the neuropil surrounding unstained cell bodies (asterisks). High magnification photomicrographs are taken of the left nuclei of interest (box). Note that the cartilage of the developing skull is dark due to counterstaining of the tissue and does not represent specific immunoreactivity. Scale bars = 150 μm in A, C, E, and G, and 15 μm in B, D, F, and H.

SNAP-25

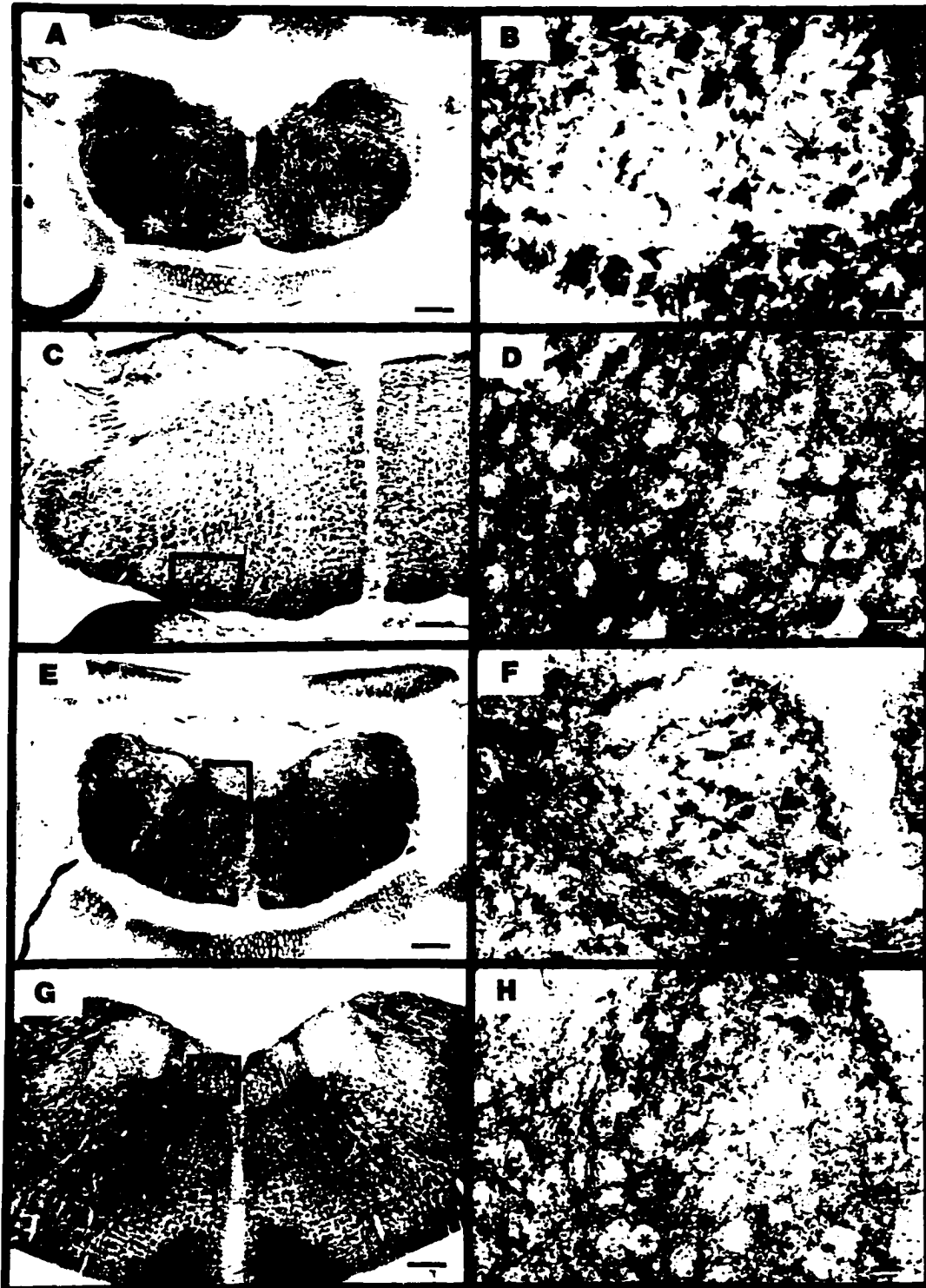
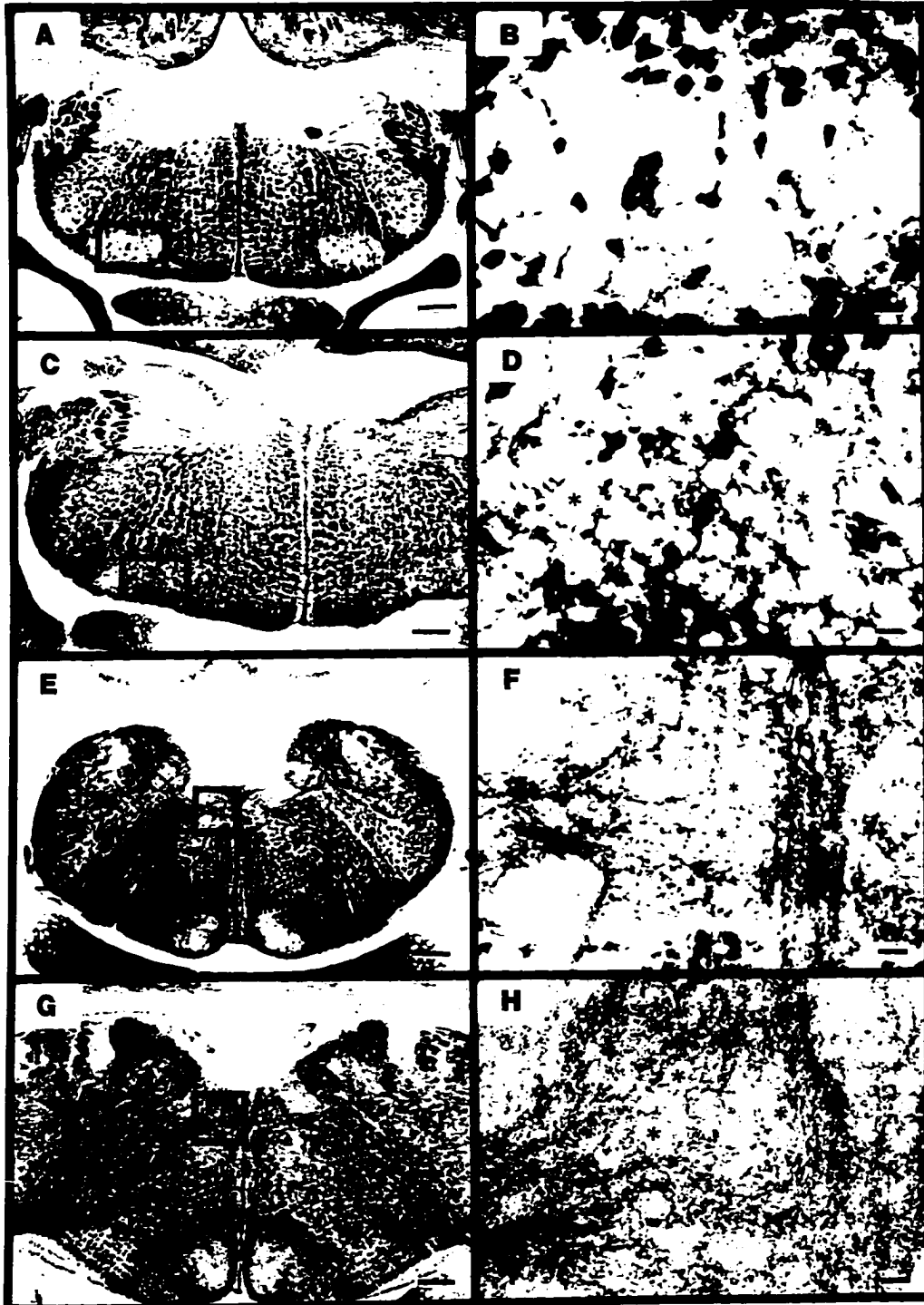


Figure 2.6. Immunoreactivity for growth-associated phosphoprotein-43 (GAP-43-IR) is absent until 9 days postnatal (PN) and fills in the facial motor nucleus by 15 PN. Photomicrographs demonstrate GAP-43-IR in the developing facial motor nucleus at 7 PN at low (A) and higher (B) magnifications and at 15 PN at low (C) and higher (D) magnifications. In contrast, GAP-43-IR is observed in the developing hypoglossal motor nucleus at all ages examined. Photomicrographs display GAP-43-IR in the developing hypoglossal motor nucleus at 5 PN at low (E) and higher (F) magnifications and at 15 PN at low (G) and higher (H) magnifications. Immunoreactivity for GAP-43 is found in the neuropil surrounding unstained cell bodies (asterisks). High magnification photomicrographs are taken of the left nuclei of interest (box). Note that the cartilage of the developing skull is dark due to counterstaining of the tissue and does not represent specific immunoreactivity. Scale bars = 150 μm in A, C, E, and G, and 15 μm in B, D, F, and H.

GAP-43



neuropil (Figs. 2.6 C,D) where growing axons would be expected.

In contrast, immunoreactivity for GAP-43 is present in the hypoglossal motor nucleus at all ages studied beginning at 1 PN (Figs. 2.6E-H). The intensity of immunoreactivity is more abundant at 5 PN (Fig. 2.6E) but appears to be less uniform when compared to that seen at 15 PN (Fig. 2.6G). In addition, the immunoreactivity appears to be restricted to the neuropil of the hypoglossal motor nucleus (Figs. 2.6F,H).

Distribution of microtubule associated proteins: PHF-1 (Tau) and MAP-2

In the facial motor nucleus, utilization of the PHF-1 antibody indicated Tau-like immunoreactivity (Tau-IR) and exhibited a similar time course of expression to that for synaptophysin and synaptotagmin. The Tau-IR was absent from the region of the facial motor nucleus through 9 PN (Fig. 2.7A,B) and immunoreactive elements appear in the nucleus over time in the same pattern as synaptophysin, synaptotagmin, SNAP-25, and GAP-43. At 15 PN (Figs. 2.7C,D), the facial motor nucleus contains immunoreactivity for Tau throughout the nucleus. Immunoreactivity appeared to be localized to the neuropil presumably where axons traveling to the facial motor nucleus would be localized and immunoreactivity was absent from the facial motoneuron cell bodies (Figs. 2.7B,D).

In contrast to the pattern of expression of Tau-IR in the facial motor nucleus, immunoreactivity in the hypoglossal motor nucleus was observed at all ages beginning on 1 PN (Figs. 2.7E,G). The immunoreactivity appeared to be localized to the neuropil of the hypoglossal motor nucleus and was absent from the hypoglossal motoneuron cell bodies (Figs. 2.7F,H).

MAP-2-like immunoreactivity (MAP-2-IR) revealed a different pattern of expression in the facial motor nucleus. The MAP-2-IR was present in the facial motor nucleus at all ages investigated beginning at 1 PN (Figs. 2.8A,C). Specifically, at higher magnification immunoreactivity was noted in the neuropil and weakly in the cell bodies of the motoneurons throughout the extent of the nucleus (Figs. 2.8B,D).

Figure 2.7. Photomicrographs demonstrating Tau-like immunoreactivity (Tau-IR) in the developing facial motor nucleus. The developing facial motor nucleus does not contain Tau-IR until 9 days postnatal (PN). Immunoreactivity is located throughout the nucleus by 15 PN following a similar time course of expression to that of synaptophysin, synaptotagmin, SNAP-25, and GAP-43.

Photomicrographs of a 5 PN opossum at low (A) and higher (B) magnifications and a 15 PN opossum at low (C) and higher (D) magnifications are shown. In addition, Tau-IR follows a similar time course of expression to synaptophysin, synaptotagmin, SNAP-25, and GAP-43 in the developing hypoglossal motor nucleus. At all ages examined Tau-IR is present in the hypoglossal motor nucleus as observed at 5 PN at low (E) and higher (F) magnifications and 15 PN at low (G) and higher (H) magnifications.

Immunoreactivity for Tau is found in the neuropil surrounding unstained cell bodies (asterisks). High magnification photomicrographs are taken of the left nuclei of interest (box). Note that the cartilage of the developing skull is dark due to counterstaining of the tissue and does not represent specific immunoreactivity. Scale bars = 150 μm in A, C, E, and G, and 15 μm in B, D, F, and H.

PHF-1

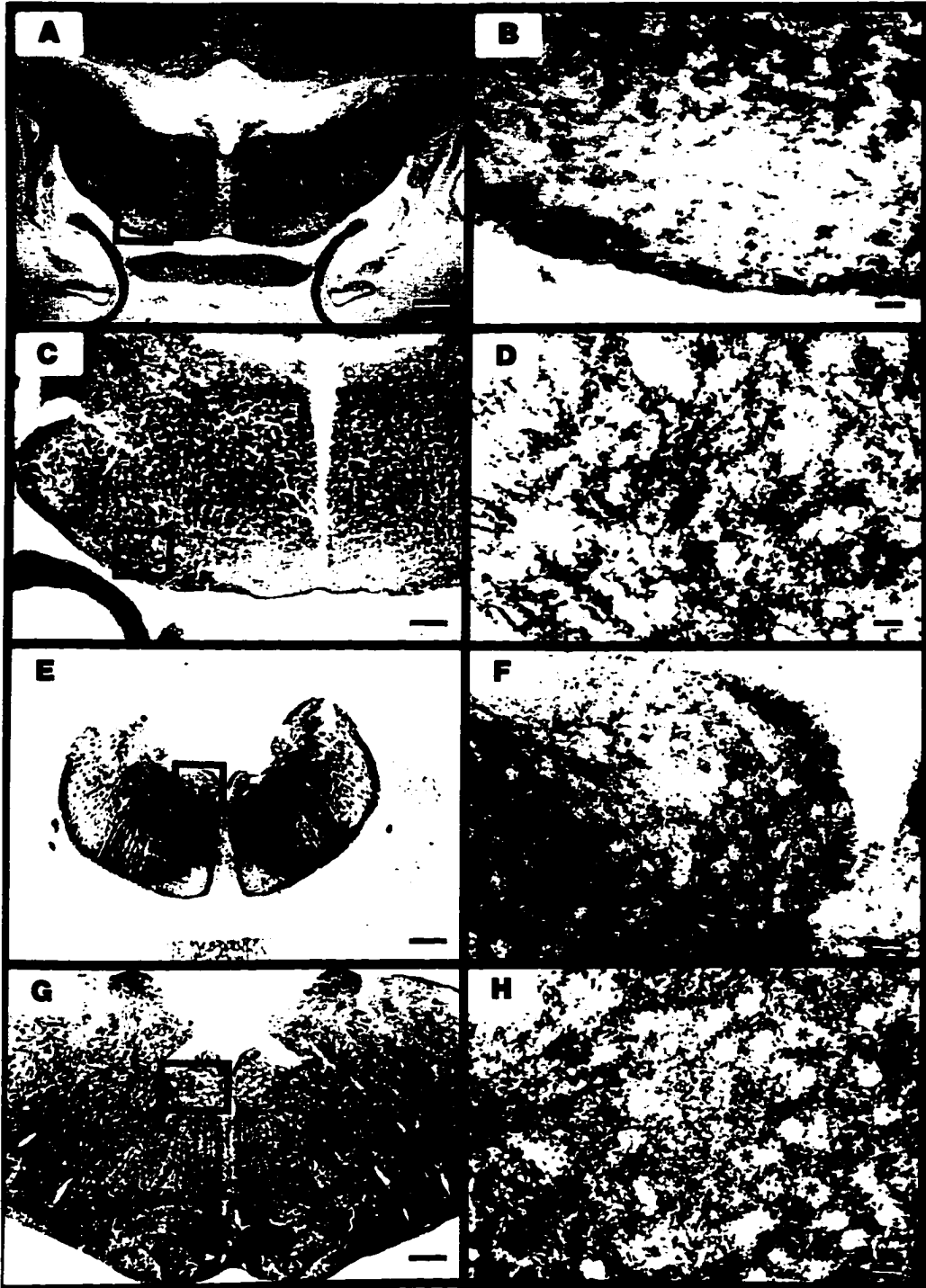
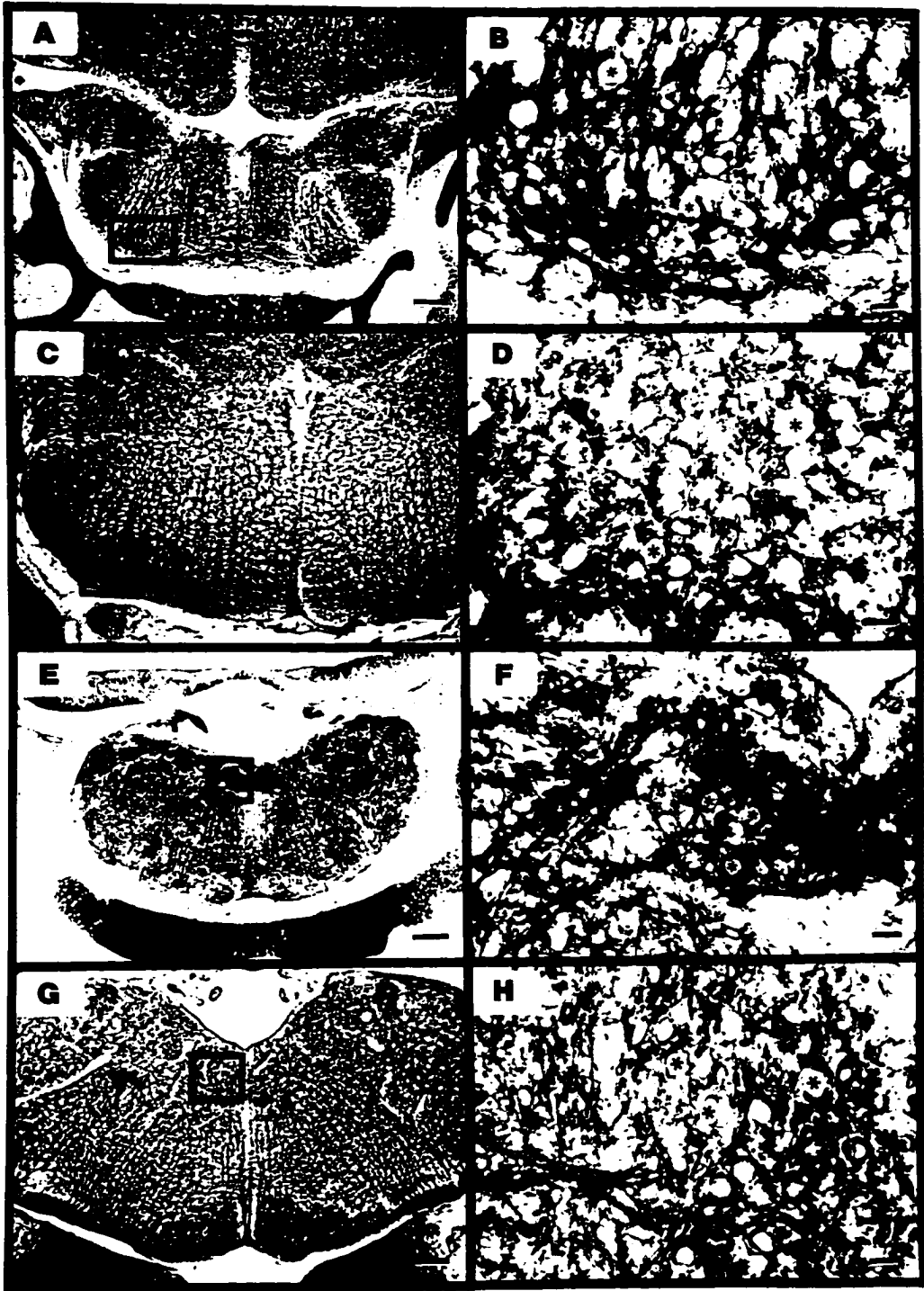


Figure 2.8. Microtubule-associated protein-2-like immunoreactivity (MAP-2-IR) in the developing facial motor nucleus and hypoglossal motor nucleus is observed from the day of birth (1 PN). Immunoreactivity for MAP-2 in the developing facial motor nucleus is shown photomicrographs at 5 PN at low (A) and higher (B) magnifications and at 10 PN at low (C) and higher (D) magnifications. Photomicrographs also display MAP-2-IR in the developing hypoglossal motor nucleus at 5 PN at low (E) and higher (F) magnifications and at 10 PN at low (G) and higher (H) magnifications. Immunoreactivity for MAP-2 is found in the neuropil surrounding weakly and/or unstained cell bodies (asterisks). High magnification photomicrographs are taken of the left nuclei of interest (box). Note that the cartilage of the developing skull is dark due to counterstaining of the tissue and does not represent specific immunoreactivity. Scale bars = 150 μm in A, C, E, and G, and 15 μm in B, D, F, and H.

MAP-2



Similarly in the hypoglossal motor nucleus immunoreactivity for MAP-2 was present at all ages studied (Figs. 2.8E,G) and was observed to be localized within the neuropil and weakly in the cell bodies of the motoneurons throughout the nucleus (Figs. 2.8F,H).

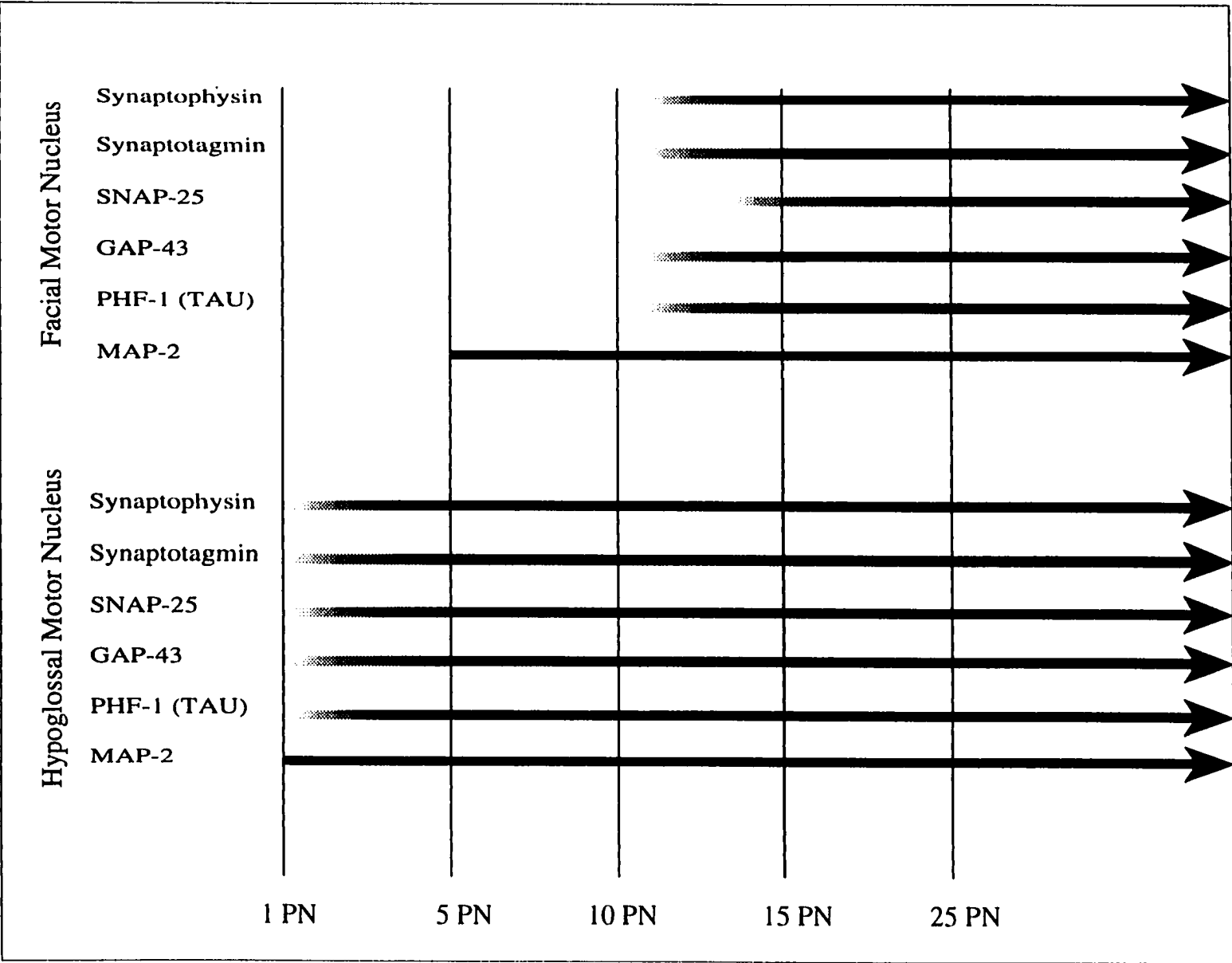
Discussion

Temporal Expression of Developmental Markers

Immunohistochemical analysis revealed the distribution of markers of synaptogenesis in the developing facial motor nucleus and hypoglossal motor nucleus in the Brazilian opossum brain (Fig. 2.9). Specifically, the two synaptic vesicle-associated proteins, synaptophysin and synaptotagmin, showed a similar pattern and time course of expression within each of the cranial motor nuclei investigated. Immunoreactivity for each synaptophysin and synaptotagmin was absent from the facial motor nuclei until 15 PN and gradually increased after this time point. Conversely, immunoreactivity in the hypoglossal motor nuclei for both synaptophysin and synaptotagmin were observed from the day of birth. These results are interesting since previous studies have concluded that the initial expression of synaptophysin is a marker of synaptogenesis. Quantitative immunoblotting for synaptophysin has demonstrated an 80-fold increase in protein levels from birth to adulthood, following a time course of accumulation that suggests that the initial synaptophysin expression is correlated with synaptogenesis (Knaus et al., 1986). In addition, immunoreactivity for synaptophysin is expressed in virtually all mature nerve terminals (Navone et al., 1986). Before cell to cell contact occurs, both synaptophysin and synapsin I are particularly concentrated in the distal axon and in growth cones (Fletcher et al., 1991). Due to these results, our observations indicate that there maybe a differential time course of synaptogenesis in the facial and hypoglossal motor nuclei.

In addition to labeling synaptic vesicle-associated proteins, antibodies against GAP-43 have been utilized as a marker of axonal growth and synaptogenesis (Dani et al., 1991). In the Brazilian opossum, GAP-43-IR follows a similar time course of expression as synaptophysin, synaptotagmin, and Tau during development of the facial motor nucleus. GAP-43-IR is absent

Figure 2.9. Time line summarizing immunoreactivity for synaptophysin, synaptotagmin, SNAP-25, GAP-43, Tau, and MAP-2 binding in the developing facial motor nucleus (FMN) and hypoglossal motor nucleus (HMN) of the Brazilian opossum. Intensity of the line correlates to the relative amount of immunoreactivity in each of the motor nuclei at that specific postnatal age (ie. first day of postnatal life = 1 PN).



in the newborn facial motor nucleus and appears by 15 PN. In addition, GAP-43-IR is similar in expression to immunostaining for synaptophysin, synaptotagmin, and Tau in the hypoglossal motor nucleus during the first two weeks of postnatal life in the Brazilian opossum. Previous studies indicate that GAP-43-IR and synaptophysin-IR have a similar pattern of expression (Masliah et al., 1991).

Paired helical filaments contain Tau (*t*) as its principal constituent (and all of the normal *t* isoforms). Phosphorylation of normal *t* is reported to be responsible for PHF properties (Greenberg et al., 1992) and is present in developing brains but not in the brains of adults. Tau immunoreactivity, which defines axons (Greenberg et al., 1992; Nelson et al., 1993), revealed a similar time course of expression to that for synaptophysin and synaptotagmin within each motor nucleus investigated. Our results with this Tau antibody indicate that axons were not in the facial motor nucleus region until after 10 PN, with an increasing amount after that period. However, axons were present in the hypoglossal motor nucleus from day one of postnatal life.

Immunoreactivity for Tau is transiently expressed by immature neurons during development (Pope et al., 1994) and is not found in mature neurons (Pope et al., 1993). We believe that this expression of Tau during development explains why immunoreactivity for Tau is not seen in the region of the developing facial motor nucleus from 1 to 15 PN. Specifically, we propose that since the facial motoneuron axons appear to innervate their targets by 1 PN they must have completed their transition from “immature” to “mature” neurons losing Tau expression. The delayed onset of immunoreactivity for Tau must be from the incoming neurons that innervate the facial motor nucleus and do not reach these neurons until 15 PN. The labeling of the afferent projections is consistent with the other markers of synaptic vesicle-associated proteins, synaptic membrane proteins, and growth cone-associated proteins.

Our results for immunohistochemistry utilizing the markers of synaptic vesicle-associated proteins and axons have the same time pattern of expression for the facial motor

nucleus. With the first appearance of the markers of synaptic vesicles in the neuropil, these results indicate that axon terminals are developing in the region of the facial motor nucleus during the first 15 days of postnatal life. The SNAP-25-IR also demonstrated a similar pattern of expression but followed a slightly later time course. This result may indicate that axons grow into the facial motor nucleus (or become immunoreactive) and subsequently form synapses onto the facial motoneuron cell bodies or dendrites.

Current models for regulated vesicle fusion and exocytosis in eukaryotic cells include both synaptotagmin and SNAP-25 as integral components during synaptic vesicle exocytosis (O'Connor et al., 1994; Bark and Wilson, 1994). The presence of immunoreactivity for synaptic vesicle-associated proteins, synaptic membranes, growth cones, and microtubule-associated proteins would suggest that synapses are formed after day 15 PN. While the absence of immunoreactivity cannot exclude synapses from the facial motor nuclei, two studies demonstrate that synapses devoid of immunoreactivity would have decreased function. Mutants with a deficiency for synaptotagmin are lacking excitation produced secretion at the synapse (Broadie et al., 1994; Geppert et al., 1994). Synapses are present in these mutants but only spontaneous activity exists at those synapses. To confirm and extend our observations, electron microscopy of the developing facial motor nucleus is being conducted.

Development of the Facial and Hypoglossal Motor Nuclei

Neurogenesis of the facial and hypoglossal motor nuclei have been investigated in detail in the neonatal rat. The facial motor nucleus has a neurogenic gradient with the rostral neurons being "older" and the caudal neurons being "younger" (reviewed in Altman and Bayer, 1995a). Specifically the rostral facial motoneurons are generated on embryonic days 12 (E12; 40%) and 13 (60%) while the caudal facial motoneurons are generated on E12 (10%), E13 (85%), and E14 (5%). Neurogenesis of the hypoglossal motor nucleus occurs on E11 (10%) and E12 (90%). Thus, the hypoglossal motoneurons are formed slightly earlier than the facial motoneurons.

Migration of facial motoneurons in the embryonic rat is also well known. After completion of neurogenesis of facial motoneurons on E13 or E14, the growth of efferents precedes the perikaryal migration through the brainstem which occurs at E15 (Altman and Bayer, 1982; Altman and Bayer, 1995b). Perikaryal migration of facial motoneurons takes an initial course directed at a right angle away from their efferent fibers. This complex pattern of migration differs dramatically from that of neurons migrating to the trigeminal nuclei. Hypoglossal motoneurons have a limited migration after neurogenesis from the fourth ventricle to the adjacent location of the hypoglossal motor nucleus.

Temporal Expression of Function

Previous reports using retrograde tract tracing of facial motoneurons in the neonatal rat indicate that the facial motoneurons innervate their respective target muscles by as early as 5 PN (Yokosuka and Hayashi, 1992; Senba et al., 1987). In the mouse, the facial motoneurons are connected to their target muscles by embryonic day 17, before the reduction of motoneurons through cell death occurs (Ashwell and Watson, 1983). We have obtained preliminary results indicating that facial motoneurons take up peripherally injected cholera toxin from their target muscles by 1 PN and that the morphology of the facial motor nucleus is adult-like by 5 PN (J. J. Swanson et al., 1995). In addition, we have preliminary results demonstrating that Brazilian opossum pups as young as 10 PN can regulate the quantity of milk ingested (M. C. Kuehl-Kovarik and C. D. Jacobson, unpublished observations).

In the neonatal rat, facial motoneurons have been shown to transiently express a number of compounds and receptors. Tribollet and coworkers (1991) have reported a transient expression of vasopressin binding sites between E17 and 19 PN in the facial nucleus of the rat. In extracellular recordings of facial motoneurons, the application of arginine vasopressin resulted in neuronal excitation (acting through a V₁ type receptor) indicating formation of functional postsynaptic receptors and second messenger systems. In addition, we have reported a transient expression of cholecystokinin (CCK) binding sites in the facial motor

nucleus of the Brazilian opossum (Kuehl-Kovarik et al., 1993a) and neonatal rat (Kuehl-Kovarik and Jacobson, 1995). Finally, Yokosuka and coworkers (1992) have described estrogen-receptor-like-immunoreactivity being transiently expressed in the facial motor nucleus of rat pups from postnatal day 1 through 11. Consistent with the receptor studies, the peptidergic and aminergic innervation of the facial motor nucleus in the neonatal rat shows an increase during the first week of postnatal life, with an established innervation pattern appearing by the tenth postnatal day (Senba et al., 1985).

Thus, many receptors are transiently expressed, although, the significance of this transient expression of the receptors is not known. Taken together with the results presented in this paper, we believe that the transient expression of receptors in the facial motor nucleus may demonstrate that the facial motor nucleus is physiologically active and the receptors may be regulating the activity of facial motoneurons independent of synaptic connections that are simultaneously being made during the postnatal period.

Summary

Characterization of synaptogenesis in the facial motor nucleus and hypoglossal motor nucleus provides a unique opportunity to study changes in motoneuron function before, during, and after synaptic formation. Our findings indicate that the facial motor nucleus, in contrast to the hypoglossal motor nucleus, does not have a synaptic or "classical" innervation during postnatal brain morphogenesis. Synaptogenesis appears following a time when facial muscle movement takes place. Thus, there is a delayed maturation of the facial motor nucleus circuitry that may allow for an additional layer of plasticity in the regulation of the neuromuscular control of food intake. Further, these results suggest that facial motor nucleus function/activity is regulated in a novel or distinct manner as compared to that of the hypoglossal during brain development.

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**CHAPTER THREE. DEVELOPMENT OF THE FACIAL AND
HYPOGLOSSAL MOTOR NUCLEI IN THE NEONATAL
BRAZILIAN OPOSSUM BRAIN**

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Abstract

The development of the facial and hypoglossal motor nuclei were examined in the neonatal Brazilian opossum (*Monodelphis domestica*), a marsupial in which postnatal central nervous system development has been well characterized. In this study, we utilized postnatal injection of the retrograde tracer cholera toxin subunit B (CtB) to characterize the formation of the facial and hypoglossal motor nuclei in the developing neonatal opossum brainstem. Injections of CtB were made into the cheek / lip region or tongue of opossum pups to retrogradely label the facial or hypoglossal motor nuclei, respectively. Following a two hour survival time, facial motoneurons in newborn opossum pups (1 PN) exhibited CtB labeling, with their cell bodies were essentially localized near the developing cranial abducens nucleus. At 3 and 5 PN, following a forty-eight hour survival time, CtB labeled facial motoneurons were observed in and migrating to the region of the adult facial motor nucleus in the rostral medulla. Between 7 and 10 PN, almost all facial motoneurons had migrated to their destination within the facial motor nucleus. Hypoglossal motoneurons also exhibited CtB labeling from 1 PN, however, their cell bodies were localized within the hypoglossal motor nucleus at the

earliest age examined. Double label studies, to examine guidance of facial motoneurons during migration, demonstrated that CtB labeled facial motoneurons are in close proximity to vimentin-like immunostained radial glial fibers during migration. These results suggest: 1) the migration of facial motoneurons to the facial motor nucleus is a postnatal event, 2) that efferent projections from facial and hypoglossal motoneurons project into the peripheral region of their target muscles from the day of birth, and 3) facial motoneurons migrate to their destination in the brainstem thereafter, in close association with radial glial fibers.

Introduction

The development of cranial motor systems within the central nervous system (CNS) consists of a complex process of events that includes neurogenesis, neuronal migration, cell death, and the establishment of both afferent and efferent connections. The investigation of the facial and hypoglossal oromotor nuclei during development will further our understanding of cranial motor system development. In order to examine the development of the facial motor nucleus (FMN) and hypoglossal motor nucleus (HMN), this study utilizes cholera toxin subunit B (CtB) as a retrograde tracer to identify facial and hypoglossal motoneurons in the developing opossum CNS.

In general, the study of the timecourse of the developing facial and hypoglossal motor systems in placental mammals would necessitate the use of *in utero* procedures. Marsupials present an attractive alternative to study neuroembryological events because they are born in an extremely immature state with a protracted postnatal period of neurogenesis [27, 31, 35, 68]. Our laboratory utilizes the Brazilian gray short-tailed opossum, *Monodelphis domestica*, a small pouchless marsupial that breeds well in the laboratory. The absence of a pouch makes the young very accessible to manipulations for *in vivo*, “ex utero” developmental studies. The ontogeny of several neurochemical systems in *Monodelphis* has been previously described by our laboratory [14-16, 21, 23, 27-30, 34, 49]. In addition, *Monodelphis* is being used to study development of multiple CNS regions including the olfactory bulbs [10, 51], visual

system [26, 60, 62, 67, 72, 80], cerebral cortex [16, 63], hypothalamus [14, 15, 20-23, 27-30, 60, 64], brainstem [36, 70], cerebellum [13], and spinal cord [11, 44, 46, 66, 73, 77].

Our recent studies have focused upon the FMN during development. Previously we have shown, using markers of synapse-associated proteins, that the FMN does not appear to receive afferent innervation until 15 PN, and mature synapses are formed between 15 and 25 PN, as compared to the HMN which appears to receive afferent innervation from birth [70]. Ultrastructure examination of the facial and hypoglossal motor nuclei during development confirms postnatal synaptogenesis within the FMN but also within the HMN with increasing age [50]. In addition, facial motoneurons transiently express cholecystokinin (CCK) binding sites between day 1 of postnatal life (1 PN) and 35 PN, disappearing by 60 PN and returning in the adult Brazilian opossum [34, 36]. Preliminary results of this study have been described in abstract form [69].

Opossum pups are born in an extremely immature state, yet already have functional systems for suckling and respiration. This study examines some of the cranial motor systems that might contribute to suckling behavior in these developing neonates. Suckling behavior would be controlled by the oromotor system comprised of three motor nuclei of cranial nerves: trigeminal (5), facial (7), and hypoglossal (12). The trigeminal motor system was not examined because developing Brazilian opossum pups lack a developed, functional dentary-squamosal joint until after 14 PN [19, 45]. Since suckling behavior begins at birth, the trigeminal component probably is not utilized for this behavior during that period before joint formation. Hypoglossal motoneurons innervating the tongue must play a crucial role in suckling mechanisms [24, 45]. In addition to the tongue musculature, the facial motor system was examined because other facial muscles might be utilized in suckling behaviors.

Motoneurons within the FMN innervate the muscles of facial expression, specifically the muscles of the ear, eyelids, nose, cheeks, lips, and the caudal digastricus [12]. Retrograde tract tracing describing the adult innervation from the FMN has been previously examined and

reported in multiple species [7, 37, 52, 53, 65, 78, 79]. In addition, these studies report that this innervation pattern forms a topographically ordered map in the FMN of the brush-tail opossum [53], cat [37, 52], monkey [79], mouse [7], and rat [78]. Throughout the studies there is general agreement that the caudal (auricular) muscles are innervated by motoneurons in the medial aspects of the FMN; dorsal (ocular) muscles are innervated by motoneurons in the dorsal aspects of the FMN; ventral muscles are innervated by motoneurons in the ventral aspects of the FMN; and the rostral (nasal) muscles are innervated by motoneurons in the lateral aspects of the FMN.

Hypoglossal motoneurons innervate the intrinsic musculature of the tongue [12]. Previous studies have demonstrated that retrogradely labeled hypoglossal motoneurons result from an injection in the tongue of developing rat pups 6 days postnatal [65]. The somatotopic organization of the HMN has been investigated by retrograde degeneration [8], tract tracing [1, 33], and cholinesterase histochemistry [38]. The studies are in general agreement that the dorsal aspects of the HMN innervate tongue retrusor muscles and the ventral aspects of the HMN innervate the tongue protrusor muscles.

In the present study, we have retrogradely labeled the motoneurons from the facial and hypoglossal motor nuclei utilizing cholera toxin subunit B. Cholera toxin subunit B (CtB) has been demonstrated as a sensitive retrograde tracer [9, 17, 39-41, 43, 74, 75, 79]. Cholera toxin subunit B was used as a retrograde tracer in this study because of its rapid speed of retrograde transport. The CtB is easily detected, immunohistochemically labeling neurons and distal dendrites with a Golgi-like appearance. The B subunit of the cholera toxin lacks the toxic effects of the intact toxin. In addition, CtB is taken up non-specifically by neurons that project to / through the injection site. With increasing survival time, CtB also labels neurons in the anterograde direction [6]. Studies have successfully utilized CtB for labeling peripheral nervous structures and cranial nerve motoneurons in the rat and monkey [1, 43, 79].

The guidance of migrating facial motoneurons is not fully understood. Previous

reports speculated that due to their migratory path facial motoneurons migrate without the aid of radial glial fibers [5]. In addition to migration, this study examined the location of migrating facial motoneurons in relation to the presence of vimentin immunostained radial glial cells during the migration of motoneurons. Radial glial cells are believed to act as physical scaffolds along which neurons can migrate (reviewed in [57-59]). In the developing cerebral cortex, cerebellum, and tectum, close associations have been observed between migrating neurons and radial glial cells [25, 54, 55, 76].

Previous studies in our laboratory have examined glial development in the Brazilian opossum brain utilizing markers for glial fibrillary acidic protein (GFAP) and vimentin (VIM; [16]). Elmquist and colleagues [16] found that GFAP and VIM are reciprocally related during development of the opossum brain. At birth, vimentin-like immunoreactivity (VIM-IR) was present throughout the brain. The majority of VIM-IR was observed in cells having a radial glial appearance and thus they considered VIM-IR to be observed within cells of glial phenotypes. Utilizing results from the previous study, this study examines the patterns of vimentin-immunostained radial glial fibers and their possible role in the migration of cranial motoneurons.

Materials and Methods

Animals

Developing Brazilian gray short-tailed opossums were obtained from a colony at Iowa State University. Animals used to start and maintain the breeding colony were obtained from the Southwest Foundation for Research and Education (San Antonio, TX). Opossums were individually housed in plastic cages, maintained at a constant temperature (26°C) with a 14:10 light-dark cycle, with food and water available ad libitum (Ferret Growth Chow, Ralston Purina, Inc. Indianapolis, IN) [35]. The animals and procedures used were in accordance with the guidelines and approval of the Iowa State University committee on animal care. To obtain pups, male and female animals were paired for breeding for 14 days and then separated.

Females were then checked daily at 1500 hours for the presence of pups (day of birth = day 1 of postnatal life; 1 PN). After a gestational period of 14 days [18, 42] pups are born in an extremely immature state, open their eyes around 30 PN, and are weaned at 60 PN [64]. At least three animals from a minimum of three different litters were used at each of the time points for the immunohistochemical study.

Retrograde labeling utilizing cholera toxin B subunit (CtB)

Brazilian Opossum pups received unilateral injections of CtB in the cheek / lip region or tongue at various timepoints after birth. Mothers were anesthetized with Metofane inhalation so they could be stabilized on their backs to expose the litter of pups. Pups were injected while attached to the dam's nipple because they do not "re-attach" if separated at early ages.

Injections of 100nl of a 1% CtB (in 0.9% saline; List Biological Laboratories, Campbell, CA), solution were administered using a 1 µl syringe (Hamilton Instruments, Reno, NV) into the cheek / lip or tongue region of the pups. Following injections the mother with her pups was returned to their cage. Following CtB injection, a survival period of 2 hours was used to retrogradely label 1 PN animals. For older animals (collected on 3, 5, 7, and 10 PN), a survival time of 48 hours following CtB injection was utilized for optimal retrograde labeling.

Tissue Preparation

Brains were then collected from the 1, 3, 5, 7, and 10 PN opossums following the appropriate survival time. Animals were anesthetized by cooling in a -15°C freezer and then decapitated. Heads were placed in Zamboni's fixative or 4% paraformaldehyde for 48 hours at 23°C. After fixation, the heads were infiltrated with 30% sucrose for 24 hours at 4°C. Brains were cut into 20 µm thick coronal sections on a cryostat (Reichert-Jung 2800N). Sections were thaw mounted onto poly-L-lysine (Sigma, St. Louis, MO) coated slides and stored at 4°C until processed for immunohistochemistry.

Immunohistochemistry

The protocol utilized for detection of CtB was a modification of that previously reported [14, 16, 21, 23, 70] by our laboratory for the Brazilian opossum brain. Briefly, slide mounted sections were rinsed, incubated with a H_2O_2 solution to remove endogenous peroxidase activity, exposed to normal serum as a blocking agent, and then incubated in goat anti-cholera toxin subunit B primary antiserum at 1:5,000 (List Biological Laboratories) for 20 hours at room temperature. After adequate washing, the tissue sections were incubated in biotinylated donkey anti-goat IgG (Jackson Laboratories, West Grove, PA; 1:1000) for 2 hours at room temperature. Sections exposed to biotinylated secondary were again rinsed and reacted with avidin-biotin complex (Vector Elite Kit, Vector Laboratories, Inc., Burlingame, CA; 1:200) at room temperature for an additional hour. After washing, the tissue sections were stained by exposing them to a substrate composed of a 3,3' diaminobenzidine tetrahydrochloride (DAB; Sigma) and H_2O_2 dissolved in 0.1 M sodium acetate. After staining for 10-18 minutes, the reaction was terminated by rinsing the slides in two successive rinses of 0.9% saline. Intensification of the staining color was achieved by adding nickel sulfate (Fisher Scientific, Pittsburgh, PA) and cobalt chloride (Sigma) to the DAB reaction step. Sections were counterstained with 0.5-1.0% neutral red (Fisher Scientific) and then dehydrated in graded alcohols, cleared in xylene and coverslipped with permount mounting media (Fisher Scientific) and analyzed and photographed with a light microscope (Axiophot, Carl Zeiss). Each run contained negative controls generated by the omission of the primary antiserum. Omission of the primary antisera resulted in no specific staining at any of the ages examined. The protocol followed for immunohistochemical detection of vimentin was previously reported by our laboratory [16].

Double label immunohistochemistry was a modification of that previously reported by our laboratory [27]. Briefly, sections were rinsed, exposed to normal rabbit serum as a blocking step, and then incubated in goat anti-cholera toxin B subunit primary antiserum at

1:5,000 (List Biological Laboratories) overnight at room temperature. Following primary antisera incubation, sections were exposed to fluorescein labeled rabbit anti-goat secondary antibodies (Vector, 1:50) for 2 hours. Sections were then blocked with normal horse serum, and incubated in mouse anti-vimentin primary antisera at 1:1000 (DAKO) overnight at room temperature. Sections were then exposed to horse anti-mouse secondary antibodies (Vector, 1:200) for 2 hours. Following secondary antisera, sections were then incubated with Texas red Avidin D (Vector, 1:50) for 2 hours. After incubation, sections were incubated with biotinylated anti-avidin (Vector, 1:100) for 2 hours. Sections were then reincubated with Texas red Avidin D. Finally sections were rinsed and coverslipped in Vectrashield (Vector).

Analysis of Immunohistochemical Tissue

Sections were examined with a Zeiss Axiophot microscope and regions containing immunoreactivity were identified and recorded on maps of coronal sections of the opossum brain [14, 16, 21, 23]. Structures were identified by reference to an atlas of the developing rat brain [3, 47, 48]. Double label experiments were viewed for analysis on a fluorescent microscope (FXA, Nikon) with fluorescein and Texas Red filter cubes.

Measurements of brainstem width, height, area, and changes in motoneuron position were made utilizing NIH Image software. Images were captured from a Nikon FXA with a Kodak Megaplug camera and imported into NIH Image running on a Apple Macintosh computer. Following calibration and image capture the NIH Image software calculated the measurements. Calculations were made from at least three brainstem sections from three animals at each of the time points investigated.

Digital Processed Figures

Figure 5 was produced digitally on a Macintosh 660AV computer. To create this figure, 2X2 slides were digitized with a slide scanner (Nikon) and transferred to Adobe Photoshop v2.5. Images were then cropped to size and saved as eps files for labeling. Aldus Freehand v3.0 was utilized for labeling the figure and the final image was printed on an

Tetronics Phaser 480 dye sublimation 300 dots per inch color printer.

Results

General Observations

Injections of CtB into the right cheek / lip region retrogradely labeled at all ages facial motoneurons localized to the right side of the brainstems (ipsilateral to the injection).

Hypoglossal motoneurons were retrogradely labeled bilaterally at all ages following a CtB injection into the tongue. Immunoreactive elements were observed filling the cell bodies and processes of the motoneurons, but not within the cell nucleus. With increasing survival time, CtB-like immunoreactivity (CtB-IR) was also observed within cells and terminals of the trigeminal sensory nucleus due to increased survival time allowing for anterograde transport. No other cells were observed labeled with CtB-IR. A description of the development of the facial and hypoglossal motor nuclei in the neonatal Brazilian opossum brain are as follows:

Facial Motor Nucleus

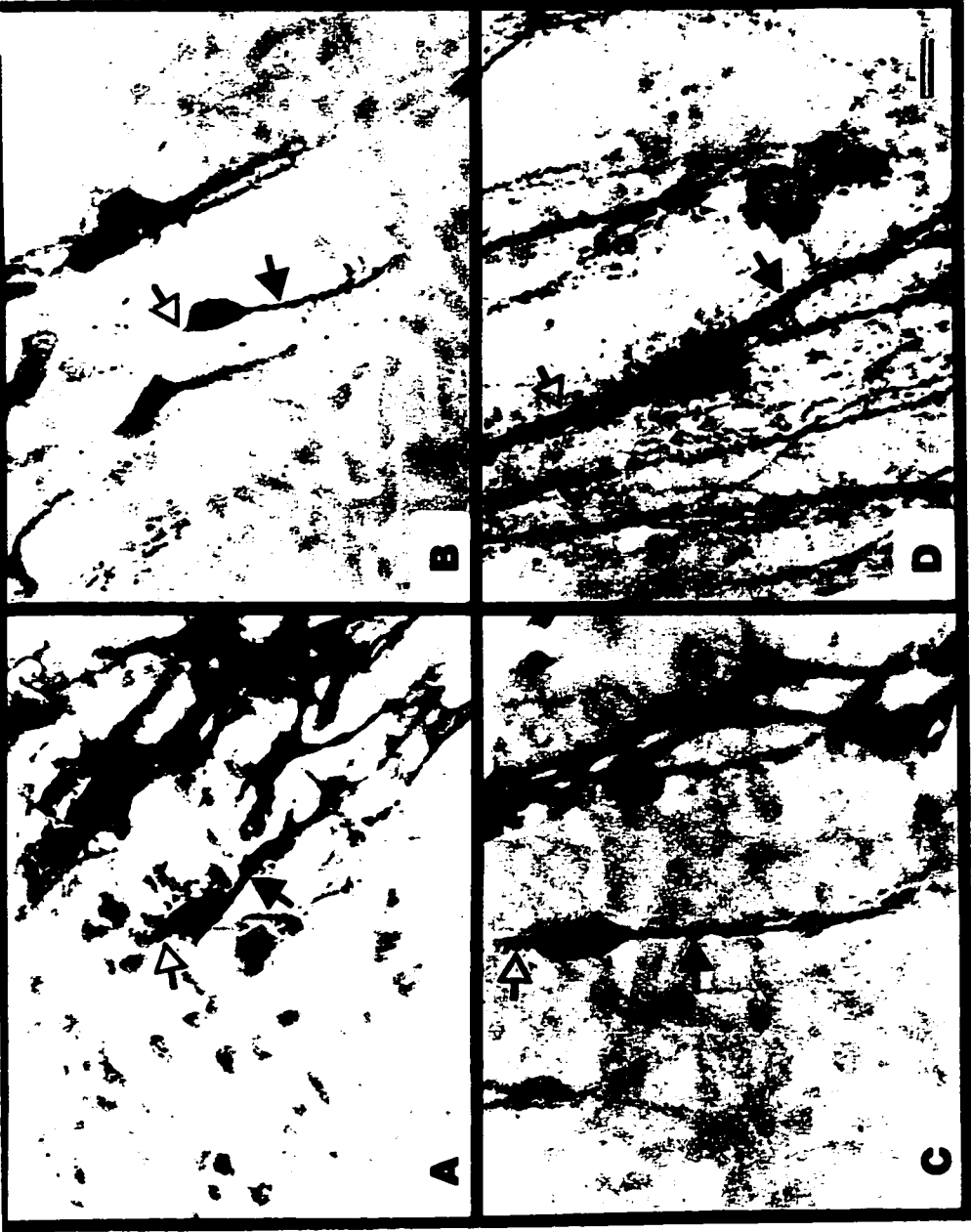
Following a two hour survival period, at 1 PN, CtB labeled facial motoneurons were observed with the majority located near the dorsal midline of the medulla (Fig. 3.1A,B). Additional labeled motoneuron cell bodies were observed in the region of the abducens cranial nucleus and distal to the developing facial nerve genu in a more lateral position. At higher magnification, facial motoneurons retrogradely labeled with CtB-IR (Fig. 3.2A) were observed to have leading processes extending from the motoneuron soma in a ventral and lateral direction (toward the presumptive future location of the FMN). At 1 PN, no motoneuron cell bodies were observed at the adult location of the FMN.

At 3 PN, following a forty-eight hour survival period, some of the facial motoneurons retrogradely labeled with CtB-IR had reached the region of the developing FMN (Fig. 3.1C,D). A majority of facial motoneurons were observed in between the dorsal portion of the brainstem and the location of the adult FMN. Motoneurons that had not yet reached the adult FMN, were observed to have leading processes (Fig. 3.2B). At higher magnification, these

Figure 3.1. A series of photomicrographs from rostral to caudal for each age, exhibiting CtB-like immunoreactivity in facial motoneurons in the developing opossum brainstem. At 1 PN (A & B), CtB retrograde labeled motoneurons were localized near the developing genu (asterisk). Motoneuron cell bodies were observed in the region of the developing facial nerve genu extending in a ventral and lateral direction. Virtually, no motoneuron cell bodies were observed at the adult location of the FMN (7). At 3 PN (C & D), some CtB labeled facial motoneurons were localized in the adult FMN. However, the majority of facial motoneurons were observed in between the developing genu and the FMN. By 5 PN (E & F), the majority of facial motoneurons have reached their destination in the FMN. Some of the CtB immunoreactive motoneurons were still observed between the genu and the FMN. By 7 PN (G & H), most of the facial motoneurons were localized to the FMN having finished migration to their destination. By 10 PN (I & J), all of the facial motoneurons were localized to the FMN, having finished migration to their destination. Note that the cartilage (white c) of the developing skull is dark due to counterstaining of the tissue and does not represent specific immunoreactivity. Abbreviations: 4V, fourth ventricle; *, developing facial nerve genu; c, cartilage. Scale bars = 100 μ m.



Figure 3.2. High power photomicrographs of CtB labeled sections at 1 PN (A), 3 PN (B), 5 PN (C), and 7 PN (D), showing the morphology of CtB-like immunoreactive facial motoneurons. Facial motoneurons displayed a bipolar shape with leading processes (filled arrowhead) and trailing processes (open arrowhead) indicating that the cell bodies are in the process of migrating to their destination. The FMN is in the direction to the lower right corner with the fourth ventricle to the top, midline to the left, lateral border to the right, and ventral border to the bottom. Scale bars = 20 μ m.



motoneuron processes labeled with CtB-IR were projecting in a ventral and lateral direction towards the location of the FMN (Fig. 3.2B).

By 5 PN, the majority of the facial motoneurons retrogradely labeled with CtB-IR had reached their destination within the FMN, following a forty-eight hour survival period (Fig. 3.1E,F). Some of the CtB-IR motoneurons were still observed in between the dorsal medulla and the adult location of the FMN. The few remaining facial motoneurons outside the location of the adult FMN were observed to have leading processes projecting in a ventral and lateral direction toward the FMN (Fig. 3.2C). In addition, the developing facial nerve was observed projecting in a dorsal and medial direction which is a similar pattern to the adult facial nerve projection.

At 7 PN, following a forty-eight hour survival period, almost all of the CtB-IR motoneurons were observed in the FMN with a few observed outside the FMN (Fig. 3.1G,H). Higher magnification examination demonstrated that a few motoneurons still outside the FMN had leading processes projecting towards the FMN (Fig. 3.2D). The developing facial nerve was observed projecting dorsal and medial toward the genu of the facial nerve. At 10 PN, following a forty-eight hour survival period, essentially all of the facial motoneurons were located within the FMN (Fig. 3.1I,J). The developing facial nerve follows a dorsal and medial pathway towards the facial nerve genu characteristic of the adult facial nerve.

Hypoglossal Motor Nucleus

At 1 PN, hypoglossal motoneurons exhibited CtB-IR labeling following injection into the tongue (Fig. 3.3A). The labeled hypoglossal cell bodies were found within the HMN. The developing hypoglossal nerve, consisting of projections from hypoglossal motoneurons, was observed projecting in a ventral and lateral direction typical for the hypoglossal nerve.

Hypoglossal motoneurons were retrogradely labeled with CtB-IR at 3 PN (Fig. 3.3B). As seen in 1 PN animals, the hypoglossal motoneurons were localized within the HMN.

Figure 3.3. A series of photomicrographs, displaying CtB-like immunoreactivity in hypoglossal motoneurons at 1 PN (A) and 3 PN (B). At both 1 and 3 PN, CtB retrogradely labeled hypoglossal motoneurons are located in the HMN. Abbreviations: 4V, fourth ventricle. Scale bars = 50 μ m.



Again the developing hypoglossal nerve, (projections from hypoglossal motoneurons) was observed projecting in a ventral and lateral direction characteristic of the adult hypoglossal nerve. Migration of hypoglossal motoneurons was not observed at any age examined.

Vimentin-like and CtB-like immunoreactivity

Vimentin-like immunoreactivity (VIM-IR) was observed in radial processes evenly spaced extending the height of the brainstem from the dorsal ventricular zone to the ventral pial surface (Fig. 3.4B,D). The dorsal aspect, near the neuroepithelium, was medial as compared to the location of the fibers on the ventral surface. Thus it appears that the position of the radial glial fibers were oriented in a dorsal to ventral direction with a medial to lateral slant.

As shown above, retrogradely labeled CtB-IR facial motoneurons were mainly seen near the facial nerve genu at 1 PN with a few motoneurons migrating toward the adult location of the FMN (Fig. 3.4A). Migrating facial motoneurons appear to be grouped with even spacing as seen for the VIM-IR radial glial fibers. At 1 PN, VIM-IR radial glial fibers were oriented in a similar direction as migrating facial motoneurons (Fig. 3.4B). In addition, as observed at 1 PN, the 5 PN facial motoneurons retrogradely labeled with CtB-IR were seen migrating in columns (Fig. 3.4C) that appear to be oriented in a similar direction as the VIM-IR radial glial fibers (Fig. 3.4D).

Double label studies, as observed at 3 PN, demonstrate columns of migrating CtB-IR facial motoneurons (FITC) are in close association with VIM-IR radial glial fibers (Texas Red) that are in between the dorsal brainstem and the adult FMN (Fig. 3.5A). Higher magnification photomicrographs demonstrate the apparent contacts between columns of CtB-IR facial motoneurons and their leading processes with the VIM-IR radial glial fibers (Fig. 3.5B). Both columns and leading processes of CtB-IR labeled facial motoneurons were observed in close proximity to VIM-IR labeled radial glial fibers.

Figure 3.4. Photomicrographs exhibiting CtB-like immunoreactivity at 1 PN (A) and 5 PN (C) and vimentin-like immunoreactivity at 1 PN (B) and 5 PN (D). Vimentin-like immunoreactive radial glial cells were oriented in the same direction (parallel arrows) as the columns of CtB-like immunoreactive facial motoneurons. Note that the cartilage (white c) of the developing skull is dark due to counterstaining of the tissue and does not represent specific immunoreactivity. Abbreviations: 4V, fourth ventricle. Scale bars = 100 μ m.

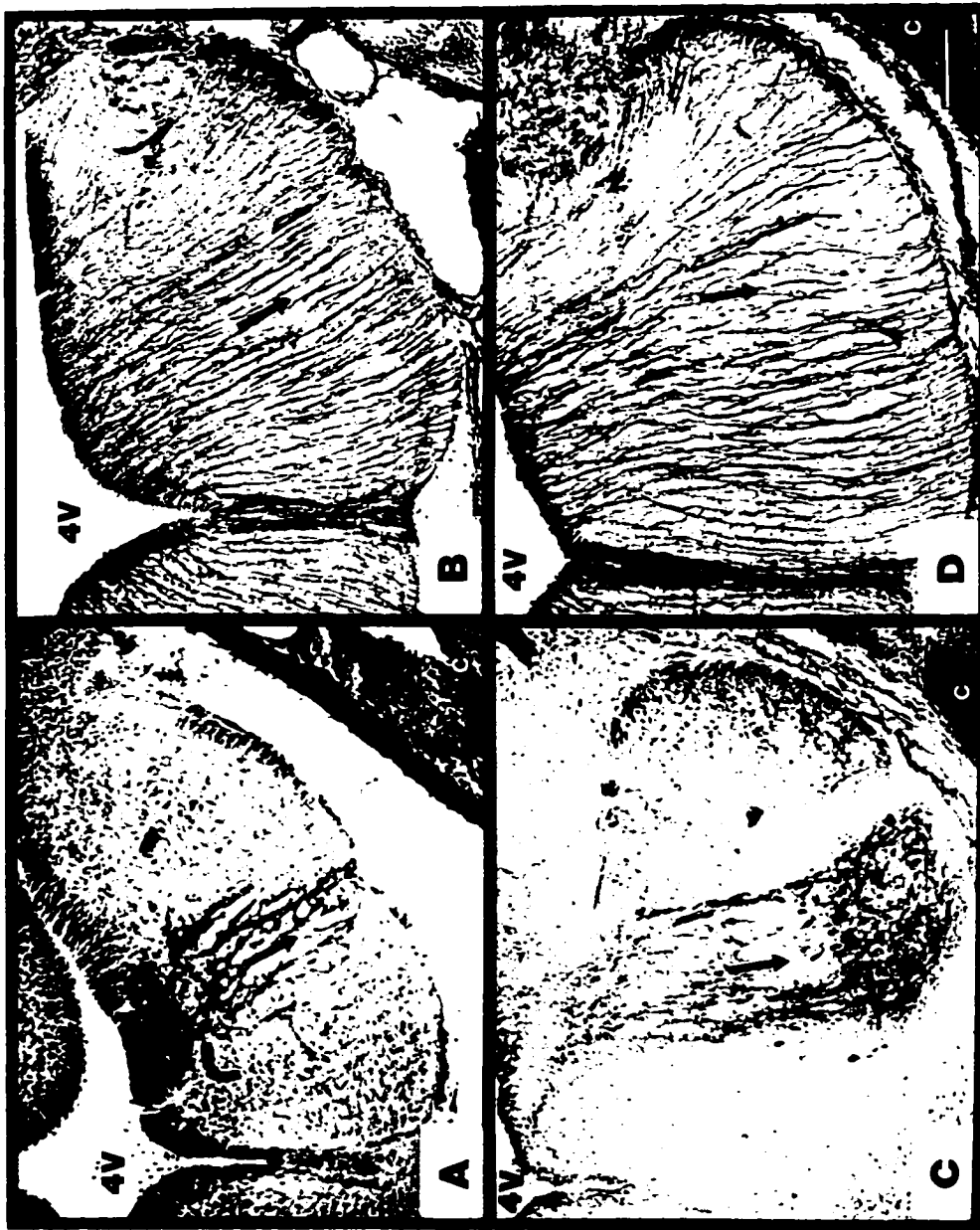


Figure 3.5. Photomicrographs demonstrating double label immunohistochemistry for CtB-IR facial motoneurons (FITC; green) and VIM-IR radial glial cells (Texas Red; red) in the 3 PN developing Opossum brain. Lower magnification photomicrographs demonstrate the VIM-IR radial glial fibers spanning the brainstem in a dorsal-ventral pattern (A). Also, the CtB-IR facial motoneurons were observed in and migrating to the FMN. In higher magnification photomicrographs columns of CtB-IR labeled facial motoneurons (arrow) were observed in close proximity of VIM-IR radial glial fibers (B). Scale bars = 100 μ m in A and 50 μ m in B.

Discussion

In the present study we have examined the development of the facial and hypoglossal motor nuclei in the neonatal Brazilian opossum brain. We have utilized postnatal injections of CtB and immunohistochemical detection of CtB for retrogradely tracing the innervation from the facial and hypoglossal motoneurons.

Retrogradely labeled facial motoneurons were localized near the developing abducens nucleus at 1 PN, within and in between the FMN and the dorsal midline of the brainstem at 3, almost all within the FMN by 5 and 7 PN, and all within the FMN at 10 PN. This protracted postnatal period of migration for the FMN differs greatly from the HMN. Retrogradely labeled hypoglossal motoneurons were observed within the HMN at all ages examined. This change in position of facial motoneurons suggests they migrate through the brainstem. Our usage of the word migration is an implied sense since we did not actually observe facial motoneurons moving.

During their migration facial motoneuron appearance was similar to other previously described migrating neurons. Rakic [32, 55, 56] previously described the appearance of migrating neurons along radial glial fibers. In this study, facial motoneurons were observed to have a leading process during their migration projecting toward the FMN and also a trailing process which in this situation would be the efferent axon from a facial motoneuron.

Migration of facial motoneurons has been well characterized in the embryonic rat [2, 5]. Following neurogenesis from the midline neuroepithelium, the growth of efferent axons precedes the perikaryal migration through the brainstem. Perikaryal migration of facial motoneurons takes an initial course directed at a right angle away from their efferent axons. This complex pattern of migration differs dramatically from and is best contrasted with trigeminal motoneurons migrating to their motor nucleus. Following neurogenesis, trigeminal motoneurons undergo perikaryal migration to the motor nucleus first and then the growth of efferent axons follows. Hypoglossal motoneurons have a limited migration after neurogenesis

from the neuroepithelium along the fourth ventricle to the adjacent location of the HMN. Hypoglossal motoneurons migrate to the region of the HMN prior to the growth of efferent projections.

Using retrograde tract tracing of facial motoneurons in the mouse, Ashwell and Watson [7] demonstrated that facial motoneurons are connected to their target muscles by embryonic day 17. In the neonatal rat, studies indicate that the facial motoneurons innervate their respective target muscles within the first five days following birth (1 PN, unpublished observations; 5 PN, [65, 81]). Thus, the expected time course for facial motoneurons to contact their respective target skeletal muscles in the rat would be embryonic, similar to the mouse. To date this study has not been specifically investigated.

Although this study did not investigate motoneuron birthdating, neurogenesis of the brainstem cranial motor nuclei appears to be a prenatal event in the Brazilian opossum (Jacobson Laboratory, unpublished observations). The time course for neurogenesis of the facial and hypoglossal motoneurons is not known for the Brazilian opossum but has been investigated in detail in the prenatal rat (reviewed in [4]). The rat FMN has a neurogenic gradient with the rostral neurons being “older” and the caudal neurons being “younger.” Facial motoneurons are generated on embryonic days 12 through 14 (E12-E14) while hypoglossal motoneurons are generated on E11 and E12. The HMN does not have a rostral to caudal gradient as observed for the FMN, however, neurogenesis of the hypoglossal motoneurons is completed slightly earlier than that for the facial motoneurons.

A comparison of the development of the facial motor system between the Brazilian opossum and the embryonic rat produces both similarities and differences. Altman and Bayer [2] observed facial motoneuron neurogenesis on E13, growth of efferent axons on E14, and perikaryal migration on days E15-E17. Neurogenesis of facial motoneurons in the opossum would be predicted to occur before birth as in the rat. Retrogradely labeling facial motoneurons with CtB at 1 PN demonstrated that the facial nerve courses laterally, exiting the brainstem

(J.J. Swanson, unpublished observations). Altman and Bayer [2] observe facial motoneuron migration on E15 through E17 which would correlate with 1 PN through 3 PN in the opossum if there is a direct day-to-day correlation. We did observe the majority of facial motoneurons were migrating on 1 PN, not ending on 3 PN but rather around 5 PN, when most facial motoneurons were at their destination.

The relative distance of migration of facial motoneurons is characterized in Table 3.1. With increasing postnatal age the distance from the midline increases from 200 μm at 1 PN to 480 μm at 7 PN. In addition, with increasing postnatal age the distance from the 4th ventricle increases from 110 μm at 1 PN to 560 μm at 7 PN. These increasing distances from the midline and 4th ventricle are summarized in a graph (Fig. 3.6) demonstrating the migration of facial motoneurons.

To determine if facial motoneuron movement is due to migration or brainstem expansion the relative rates were calculated (Table 3.2). During postnatal days 1 through 7 facial motoneurons move away from the fourth ventricle at an average rate of 87.5 μm . During the same time period the brainstem expands in height at an average rate of 65.0 μm . However, facial motoneurons move away from the midline at a rate of 48.7 μm while the brainstem expands in width at a rate of 94.3 μm . Since the expansion in width is twice the distance from the midline the adjusted expansion would be at a rate of 47.2 μm which is very similar to the rate of facial motoneuron movement. From these data we conclude that the facial motoneurons do migrate due to their movement away from the fourth ventricle at a faster rate than brainstem height expansion. However, the horizontal movement of facial motoneurons is at a similar rate of brainstem width expansion so their migration in this direction is unknown. Further statistical analysis will be performed to determine the significance of these changes in distance and rates.

Due to the complex arch of their efferent projections and migration pathway, facial motoneurons were thought to migrate to their destination without the aid of radial glial cells [5].

Table 3.1. Average width, height, and area for developing brainstems with relative distance from midline and 4th ventricle for migrating facial motoneurons (in μm).

Age (postnatal days)	Average width (μm)	Average distance from midline (μm)	Average height (μm)	Average distance from 4th ventricle (μm)	Average area (μm^2)
1 PN	1160	200	440	110	550,000
3 PN	1360	310	580	290	870,000
5 PN	1520	380	700	500	1,400,000
7 PN	1720	480	860	560	1,590,000

Figure 3.6. Graph of CtB labeled facial motoneuron location during migration. With increasing age the distance between facial motoneurons and the midline or 4th ventricle increases. These results demonstrate the migration of facial motoneurons away from their origin of neurogenesis.

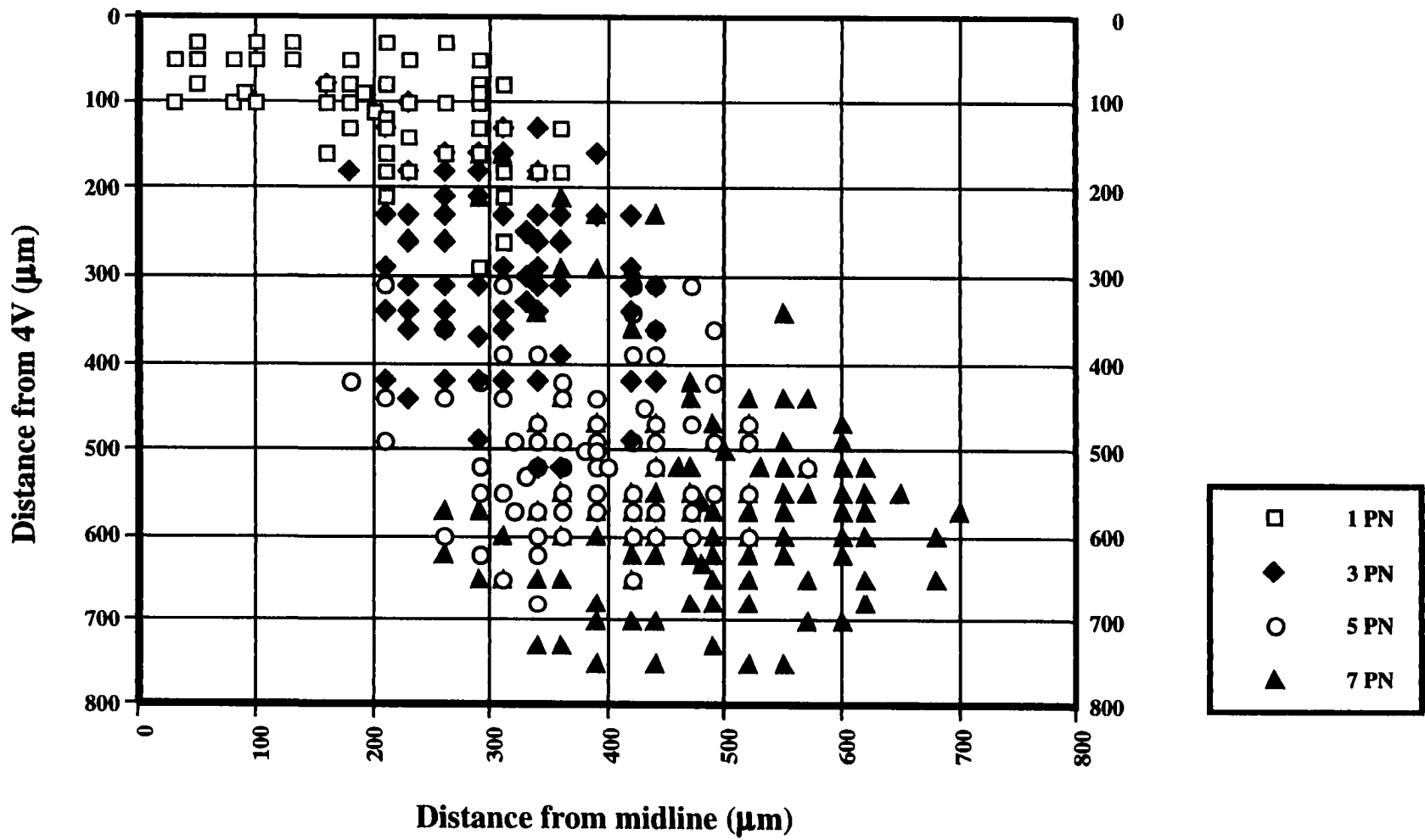


Table 3.2. Rate of facial motoneuron migration and rate of brainstem expansion (in $\mu\text{m}/\text{day}$).

Time Period (postnatal days, PN)	Rate of facial motoneuron migration from 4V ($\mu\text{m}/\text{day}$)	Rate of brainstem expansion in width ($\mu\text{m}/\text{day}$)	Rate of facial motoneuron migration from midline ($\mu\text{m}/\text{day}$)	Rate of brainstem expansion in height [1/2] ($\mu\text{m}/\text{day}$)
1-3 PN	90.0	60.0	55.0	100.0 [50.0]
1-5 PN	97.5	65.0	45.0	90.0 [45.0]
1-7 PN	75.0	70.0	46.0	93.0 [46.5]
Mean	87.5	65.0	48.7	94.3 [47.2]

Our results do not agree with their hypothesis. In addition, a previous ultrastructural study by Ruhrig and Hummel [61] reported that the perikarya of areas of facial motoneurons were in contact with radial fibers during their migration in the developing bovine brain stem. In this study we utilized double label immunostaining with CtB-IR and VIM-IR to demonstrate that facial motoneurons are in close proximity to radial glial fibers during their migration as well. These results suggest that facial motoneurons utilize radial glial fibers during their migration to their destination.

To examine facial and hypoglossal motoneuron projections, we have performed immunohistochemistry for choline acetyltransferase-like immunoreactivity (ChAT-IR) to examine the developing facial and hypoglossal nerves [71]. Preliminary results demonstrate that from the day of birth, the facial and hypoglossal nerves are ChAT-immunoreactive. At 1 PN, the facial nerve, labeled with ChAT-IR, was observed exiting the skull into the periphery, turning rostrally, and extending toward target muscles. In addition, utilizing synaptotagmin-like immunoreactivity, we have localized presynaptic terminals in target muscles of facial motoneurons. These data suggest that facial motoneurons innervate their target muscles from the day of birth. Thus facial motoneurons have innervated their target muscles during a period when their cell bodies are still migrating to their final destination within the FMN.

The development of the facial motor system is apparently completed long before birth in placental mammals. However, at birth, the facial motor system of the Brazilian opossum is still partially incomplete, with the efferents projecting to the target muscles during a period when the cell bodies are migrating to their destination. During this period when cell bodies of facial motoneurons are migrating if functional they might be expected to lack "classical" innervation and, therefore, would possibly be regulated in some other way. The transient expression of cholecystinin binding sites as reported by Kuehl-Kovarik and coworkers [34, 36] might provide for an additional level of regulation during migration.

Summary

In conclusion, we have examined the development of the facial and hypoglossal motor nuclei in the Brazilian opossum brain utilizing the retrograde tracer cholera toxin subunit B. Facial motoneurons in newborn opossum pups (1 PN) exhibited CtB-IR and their cell bodies were localized near the dorsal midline of the medulla. At 3 PN, some CtB-IR labeled facial motoneurons were observed in the FMN with a majority observed between the facial nerve genu and the FMN. By 5 PN, the CtB-IR labeled facial motoneurons were observed mostly within the FMN with a few still migrating. Between 7 and 10 PN, CtB-IR labeled facial motoneurons have reached their destination in the FMN as seen in the adult. In contrast, CtB-IR labeled hypoglossal motoneurons were observed in the HMN from 1 PN. Double label studies demonstrated that facial motoneurons labeled with CtB-IR were in apparent contact with VIM-IR labeled radial glial fibers during their migration. These results suggest that the developmental history of the facial and hypoglossal motor nuclei is different. Further, the results presented here indicate that the FMN might be able to regulate motor activity prior to completing its own embryonic development.

Acknowledgments

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**CHAPTER FOUR. FACIAL AND HYPOGLOSSAL MOTONEURON
PROJECTIONS EXTEND TO THEIR RESPECTIVE TARGET MUSCLES
IN THE NEONATAL BRAZILIAN OPOSSUM**

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Abstract

The anatomical distribution of facial and hypoglossal cranial nerves were examined in the neonatal Brazilian opossum (*Monodelphis domestica*). Efferent projections from facial and hypoglossal motoneurons were localized utilizing immunohistochemical detection of choline acetyltransferase, neurofilament, and synaptotagmin. Facial and hypoglossal motoneurons and nerves were choline acetyltransferase-like immunoreactive (ChAT-IR) on the day of birth (1 PN). At 1 PN, the facial nerve, immunostained with ChAT, was observed exiting the skull, extending rostrally, and branching in its target muscles. In addition, the caudal auricular, temporal, zygomatic, buccal, and marginal mandibular branches of the facial nerve were observed utilizing immunohistochemistry for neurofilament in whole mount preparations. The hypoglossal nerve, labeled at 1 PN with ChAT-IR, was also observed in the periphery extending toward, entering into, and branching within the tongue. In whole mount preparations at 1 PN, the hypoglossal cranial nerve was observed exiting the skull, coursing through the periphery, and entering into the tongue. Further, at 1 PN, presynaptic terminals

were localized in target muscles innervated by either facial or hypoglossal motoneurons, using immunohistochemistry for synaptotagmin-like immunoreactivity. We have previously shown that facial motoneurons in the Brazilian opossum are actively migrating for the first postnatal week. However, data presented here suggest that facial and hypoglossal motoneurons have contacted, and may innervate their target muscles from the day of birth; a time during which facial motoneurons are still migrating.

Introduction

The oromotor system consists of the trigeminal (5), facial (7), and hypoglossal (12) motor nuclei and their associated cranial nerves [85]. Facial motoneuron projections innervate the muscles of facial expression, specifically the muscles of the ear, eyelids, nose, cheeks, lips, and the caudal digastricus [7]. Hypoglossal motoneuron projections innervate the intrinsic musculature of the tongue. The motoneuron component of the trigeminal cranial nerve innervates the muscles utilized in mastication.

Cell bodies of facial motoneurons are located within the facial motor nucleus (FMN) found in the ventral-rostral medulla. Previous studies have demonstrated that the FMN of mammals consists of discrete subnuclei with an orderly topographical representation of facial nerves and musculature within the FMN of the adult cat [11, 53], dog [53, 89], guinea pig [53], monkey [93], mouse [2], opossum (Brush-tailed [66], and North American [14]) and rat [30, 41, 46, 48, 53, 73, 92]. In general, those studies reported that, the caudal (auricular) musculature is innervated by the motoneurons in the medial aspects of the FMN, the dorsal (ocular) musculature is innervated by motoneurons in the dorsal aspects of the FMN, ventral musculature is innervated by motoneurons in the ventral aspects of the FMN, and rostral (nasal) musculature is innervated by motoneurons in the lateral aspects of the FMN. In addition, facial nerve development has been documented in eutherian mammals [3, 4, 25, 56, 57, 69]. Such studies of cranial nerve development in placental mammals required the use of *in utero* procedures [3, 4] or cesarean section fixation at critical time points [25, 56, 57, 69].

Studies in neonatal rats reported similar results for FMN innervation patterns as seen in adult rats [39]. These studies suggest that the development of FMN efferent projections must be prenatal in rats.

A topographical organization also exists for the adult hypoglossal nucleus and has been described in the dog [9, 87], monkey [75, 88], rabbit [87], and rat [1, 42, 87]. These studies have demonstrated that motoneurons in the dorsal aspects innervate the tongue retrusor muscles and that motoneurons in the ventral aspects innervate the tongue protrusor muscles. The development of the hypoglossal cranial nerve has not been described as in depth regarding its topographical organization. Holt [31] examined the tongue musculature for acetylcholinesterase activity and reported its presence at embryonic day 14 (E14) in the rat at the light and electron microscopic level. Wragg and coworkers [95] localized the hypoglossal nerve within the tongue musculature at E14 in the rat, and by E15 tongue musculature responded to electrophysiological stimuli.

All of these studies examining the development of the facial and hypoglossal cranial nerves utilized placental mammals. Because it appears that motoneuron efferent and projection growth is prenatal, examination of the developing cranial nerve motor systems in placental mammals necessitates the use of *in utero* procedures [3, 4]. Marsupials are a useful tool that present an attractive alternative to study neuroembryological events. Marsupial young are born in an extremely immature state which might be considered almost an embryonic condition when compared to eutherian young. Marsupial neonates have a protracted period of neurogenesis that continues into the postnatal period [33, 37, 44, 79]. Yet at birth, these developing neonates must already possess functional systems for suckling and respiration. To take advantage of this developmental tool, our laboratory utilizes *Monodelphis domestica*, the Brazilian gray short-tailed opossum. *Monodelphis* is a small pouchless marsupial that breeds well under laboratory conditions. The absence of a pouch makes the young very accessible to manipulations for *in vivo*, "ex utero" developmental studies. Our laboratory has previously

described the ontogeny of several neurochemical systems in *Monodelphis* [19-21, 23, 24, 33-36, 43, 58]. In addition, several other laboratories are utilizing *Monodelphis* to study development of CNS regions including: olfactory bulbs [6, 65], visual system [27, 67, 68, 77, 84, 94], cerebral cortex [70], hypothalamus [67, 72], brainstem [91], cerebellum [15], and spinal cord [8, 50, 52, 76, 86].

Our laboratory has previously established that facial motoneurons migrate postnatally [80, 82]. Therefore, we wanted to determine if efferent development was also a postnatal event. Through the use of markers for axonal processes and presynaptic terminals, this study examined the efferent projections from facial and hypoglossal motoneurons. To accomplish this task three immunohistochemical markers were used: choline acetyltransferase, neurofilament, and synaptotagmin. Choline acetyltransferase is the enzyme that produces acetylcholine (ACh) from acetyl Co-A and choline. The neurotransmitter at neuromuscular junctions is ACh, thus choline acetyltransferase would be expected in motoneurons. Previous reports labeling motoneurons during development have successfully utilized ChAT immunohistochemistry [5, 10, 16-18, 32, 60-64]. Immunoreactivity for ChAT has been observed in cell bodies of sensory neurons, however, ChAT immunoreactivity has been only in the fibers from motoneurons. Neurofilaments play a role in maintaining cytoskeleton integrity [38]. Immunohistochemistry for neurofilament allows for visualization of axonal outgrowth [28]. Visualization of the peripheral nervous system is possible utilizing whole mount immunohistochemistry. Without utilizing whole mount immunohistochemistry, studies require three-dimensional reconstruction of serial sections for visualization of neurite processes and peripheral nerves. Synaptotagmin has been localized within synaptic vesicle membranes and is thought to be needed in the secretory process [78, 90]. Thus synaptotagmin, as a marker of synapse existence, was used to localize presynaptic neuromuscular junctions. Preliminary results of this study have been previously reported in abstract form[83].

Materials and Methods

Animals

Developing Brazilian gray short-tailed opossums were obtained from a colony at Iowa State University. Animals used to start and maintain the breeding colony were obtained from the Southwest Foundation for Research and Education (San Antonio, TX). Opossums were individually housed in plastic cages, maintained at a constant temperature (26°C) with a 14:10 light-dark cycle, with food and water available ad libitum (Ferret Growth Chow, Ralston Purina, Inc. Indianapolis, IN) [44]. The animals and procedures used were in accordance with the guidelines and approval of the Iowa State University Committee on Animal Care. To obtain pups, male and female animals were paired for breeding for 14 days and then separated. Females were then checked daily at 1500 hours for the presence of pups (day of birth = day 1 of postnatal life; 1 PN). After a gestational period of 14 days [22, 49] pups are born in an extremely immature state, open their eyes around 30 PN, and are weaned at 60 PN [72]. At least three animals from a minimum of three different litters were used for the immunohistochemical studies.

Tissue Preparation

Heads were collected from 1 PN opossums. Animals were anesthetized by cooling in a -15°C freezer and then decapitated. Heads were placed in 4% paraformaldehyde for 48 hours at 4°C. After fixation, the heads were infiltrated with 30% sucrose for 24 hours at 4°C. Brains were cut into 20 mm thick coronal sections on a cryostat (Reichert-Jung 2800N). Sections were thaw mounted onto poly-L-lysine (Sigma) coated slides and stored at 4°C until processed for immunohistochemistry.

Immunohistochemistry

The protocol utilized for detection of choline acetyltransferase was a modification of the immunohistochemical protocol previously reported [19, 21, 23, 24, 81] by our laboratory for the Brazilian opossum. Briefly, slide mounted sections were rinsed, incubated with a H₂O₂

solution to remove endogenous peroxidase activity, exposed to normal donkey serum as a blocking agent, and then incubated in goat anti-choline acetyltransferase primary antiserum at 1:300 (AB144; Chemicon) for 20 hours at room temperature. After adequate washing, the tissue sections were incubated in biotinylated donkey anti-goat IgG (Jackson Laboratories; 1:1000) for 2 hours at room temperature. Sections were again rinsed and reacted with avidin-biotin complex (Vector Elite Kit; 1:200) at room temperature for an additional hour. After washing, the tissue sections were stained by exposing them to a substrate composed of a 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma) and H_2O_2 in 0.1 M sodium acetate. The reaction of glucose oxidase (Sigma) with the addition of β -D-glucose (Sigma) and 0.1% NH_4Cl yielded the H_2O_2 for the DAB. After staining for 10-18 minutes, the reaction was terminated by rinsing the slides in two successive rinses of 0.9% saline. Intensification of the staining color was achieved by adding nickel sulfate (Fisher Scientific) and cobalt chloride (Sigma) to the DAB reaction step. Sections were counterstained with 0.5-1.0% neutral red (Fisher Scientific) and then dehydrated in graded alcohols, cleared in xylene and coverslipped with permount mounting media (Fisher Scientific) and analyzed and photographed with a light microscope (Axiophot, Carl Zeiss). Each run contained negative controls generated by the omission of the primary antiserum. Omission of the primary antisera resulted in no specific staining at any of the ages examined. The detection of synaptotagmin utilized a previously published protocol that we have successfully used in the Brazilian opossum [81].

Whole Mount Immunohistochemistry

The protocol utilized for whole mount immunohistochemistry was a modification of that previously established for the Brazilian opossum ([47], J. M. Luque and J. G. Nicholls, personal communication). This protocol is similar to others previously reported [12, 29, 40, 71, 74]. Briefly, heads were collected from 1 PN opossums. Animals were anesthetized by cooling in a $-15^\circ C$ freezer and then decapitated. Heads were dissected and placed in Dent's fixative (4 parts methanol, 1 part dimethyl sulfoxide [DMSO]) for 2 days. Following fixation

the heads were incubated in Dent's bleach (2 parts Dent's fixative and 1 part 30% hydrogen peroxide) for 3 days. Heads were dehydrated in absolute methanol twice for 30 minutes and then stored at -20°C overnight (or for extended storage). Following freezing (storage) the heads were thawed and washed in tris buffered saline (TBS, pH 7.6). Heads were incubated in primary antisera, mouse anti-neurofilament (2H3, 1:25), diluted in normal calf serum with 20% DMSO overnight. The monoclonal neurofilament (165 kDa) antibody developed by T. M. Jessel and J. Dodd [13] was obtained from the Developmental Studies Hybridoma Bank maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, under contract NO1-HD-7-3263 from the NICHD. After adequate washing (5 times for 1 hour in TBS), the heads were incubated in peroxidase-conjugated donkey anti-mouse IgG (Jackson Laboratories; 1:500) overnight. After washing, the heads were stained by exposing them to a substrate composed of a 3,3' diaminobenzidine tetrahydrochloride (DAB; Sigma) and H_2O_2 in 0.1 M sodium acetate. The reaction of glucose oxidase (Sigma) with the addition of β -D-glucose (Sigma) yielded the H_2O_2 for the DAB. Intensification of the staining color was achieved by adding nickel sulfate (Fisher Scientific) to the DAB reaction step. After staining for 10-18 minutes, the reaction was terminated by rinsing the slides in two successive rinses of methanol. Heads were then dehydrated in methanol and cleared in 1 part benzyl alcohol, 2 parts benzyl benzoate solution (BABB).

Analysis of Immunohistochemical Tissue

Sections were examined with a Zeiss Axiophot microscope and regions containing immunoreactivity were identified by reference to an atlas of the developing rat brain and periphery [54, 55]. For whole mount analysis heads were observed and photographed with a dissecting microscope (SMZ-U, Nikon) in BABB solution.

Digital Processed Figures

Figure 2C was produced digitally on a Macintosh 660AV computer. To create this figure, drawings were digitized with a flatbed scanner (Hewlett Packard Scanjet 4c) and

transferred to Adobe Photoshop v2.5. Images were then cropped to size and saved as eps files for labeling. Aldus Freehand v3.0 was utilized for labeling the figure and the final image was printed on a laser printer (Hewlett Packard Laserjet 4MP).

Results

Projections from Facial Motoneurons

Choline acetyltransferase-like immunoreactivity (ChAT-IR) labeled the facial nerve from the day of birth (1 PN; the earliest age we examined). The ChAT-IR was observed in efferent projections from facial motoneurons coursing laterally and exiting the rostral medulla (Fig. 4.1A). In the periphery, the facial nerve was seen exiting the skull (Fig. 4.1B). After exiting the skull, the facial nerve was seen to curve ventrally before turning and projecting rostrally (Fig. 4.1B-D).

Whole mount immunohistochemistry for neurofilament improved visualization of the facial nerve in the periphery (Fig. 4.2). At 1 PN, the facial nerve labeled with neurofilament-like immunoreactivity was observed exiting the skull, curving ventrally before turning and projecting rostrally (Figs. 4.2A,B). Several branches of the facial nerve were observed at 1 PN such as: posterior auricular, temporal, zygomatic, buccal, and marginal mandibular (Fig. 4.2C).

Synaptotagmin-like immunoreactivity was utilized for examination of presynaptic terminal formation of facial motoneuron projections within target muscles. At 1 PN, synaptotagmin-like immunoreactivity was observed in the posterior digastric (Fig. 4.3A) and platysma (Fig. 4.3B) muscles.

Projections from Hypoglossal Motoneurons

Hypoglossal motoneurons and the corresponding cranial nerve were both immunoreactive for choline acetyltransferase on the day of birth. In the periphery, at 1 PN, the hypoglossal nerve labeled with ChAT-IR was observed entering the tongue from the ventral aspects and extending in a dorsal and medial direction (Fig. 4.4A). In horizontal sections, the

Figure 4.1. A series of photomicrographs demonstrating choline acetyltransferase-like immunoreactivity in efferent projections from facial motoneurons at 1 PN. Immunoreactivity for choline acetyltransferase was observed in the facial nerve (arrows): exiting the brainstem (A; coronal section), in the periphery (B; sagittal section), turning rostrally (C; sagittal section), and projecting rostrally (D; sagittal section). Sagittal sections (B, C, & D) are sequential with a 40 μm distance inbetween them. Note that the cartilage of the developing skull is dark due to counterstaining of the tissue and does not represent specific immunoreactivity. Abbreviations: Aq, cerebral aqueduct; bo, basioccipital bone; cb, cerebellum; FL, forelimb; msg, submandibular salivary gland; sc, superior colliculus; 4, fourth ventricle. Scale bars = 200 μm .

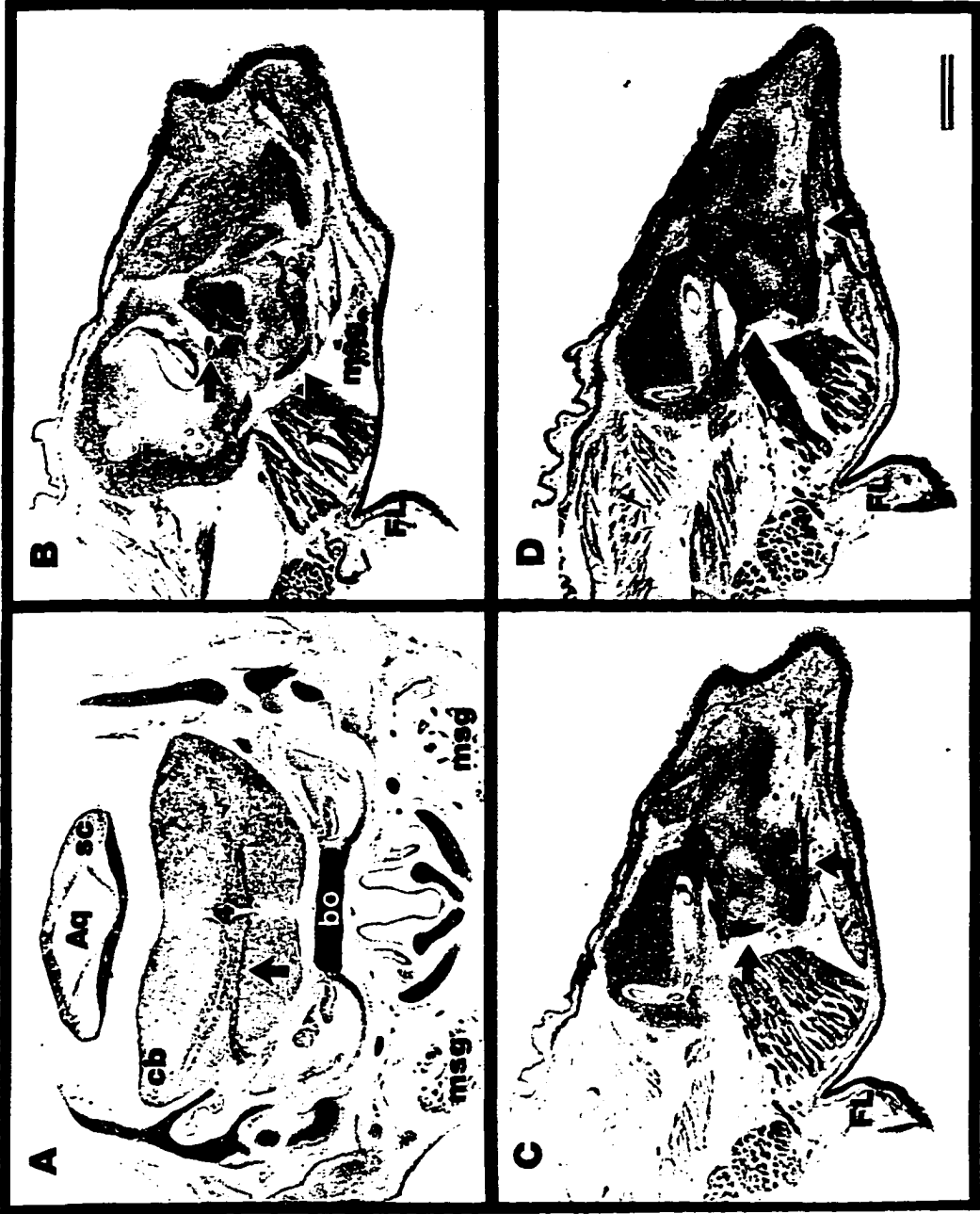


Figure 4.2. Photomicrograph (A) of whole mount immunohistochemistry for neurofilament in a 1 PN opossum head. Higher magnification photomicrograph (B) demonstrating branches of the facial nerve at 1 PN. Neurofilament-like immunohistochemistry allows visualization of the branches of the facial nerve at 1 PN. Scale bars = 200 μ m. Schematic diagram (B) of the facial nerve in the neonatal opossum head. Caudal auricular [PA], temporal [T], zygomatic [Z], buccal [Bu], and marginal mandibular [M], branches of the facial nerve can be seen in A at 1 PN demonstrating the efferent projection of facial motoneurons at this age.

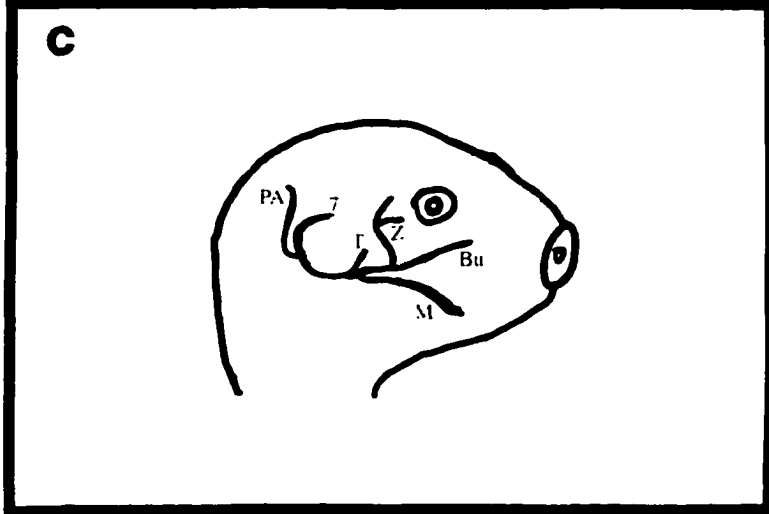


Figure 4.3. A series of photomicrographs showing synaptotagmin-like immunoreactivity in facial motoneuron efferent projections within the target muscles: posterior digastricus (A) and platysma (B) at 1 PN, respectively. Synaptotagmin-like immunoreactivity was observed in the nerve branching into the muscle (open arrowhead) and in presumptive presynaptic terminals (closed arrowhead). Abbreviations: dig, posterior digastricus muscle; mh, mylohyoideus muscle; pma, platysma muscle; sk, skin. Scale bars = 50 μ m.

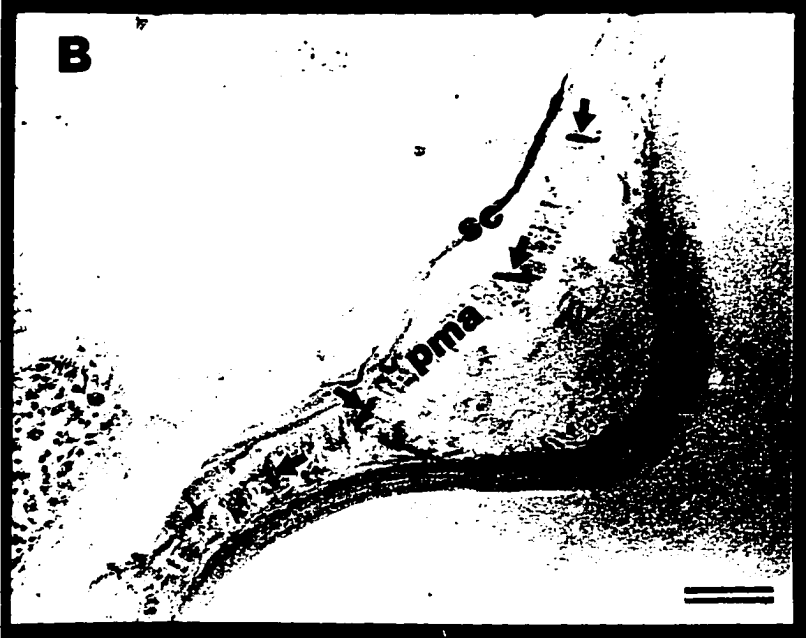
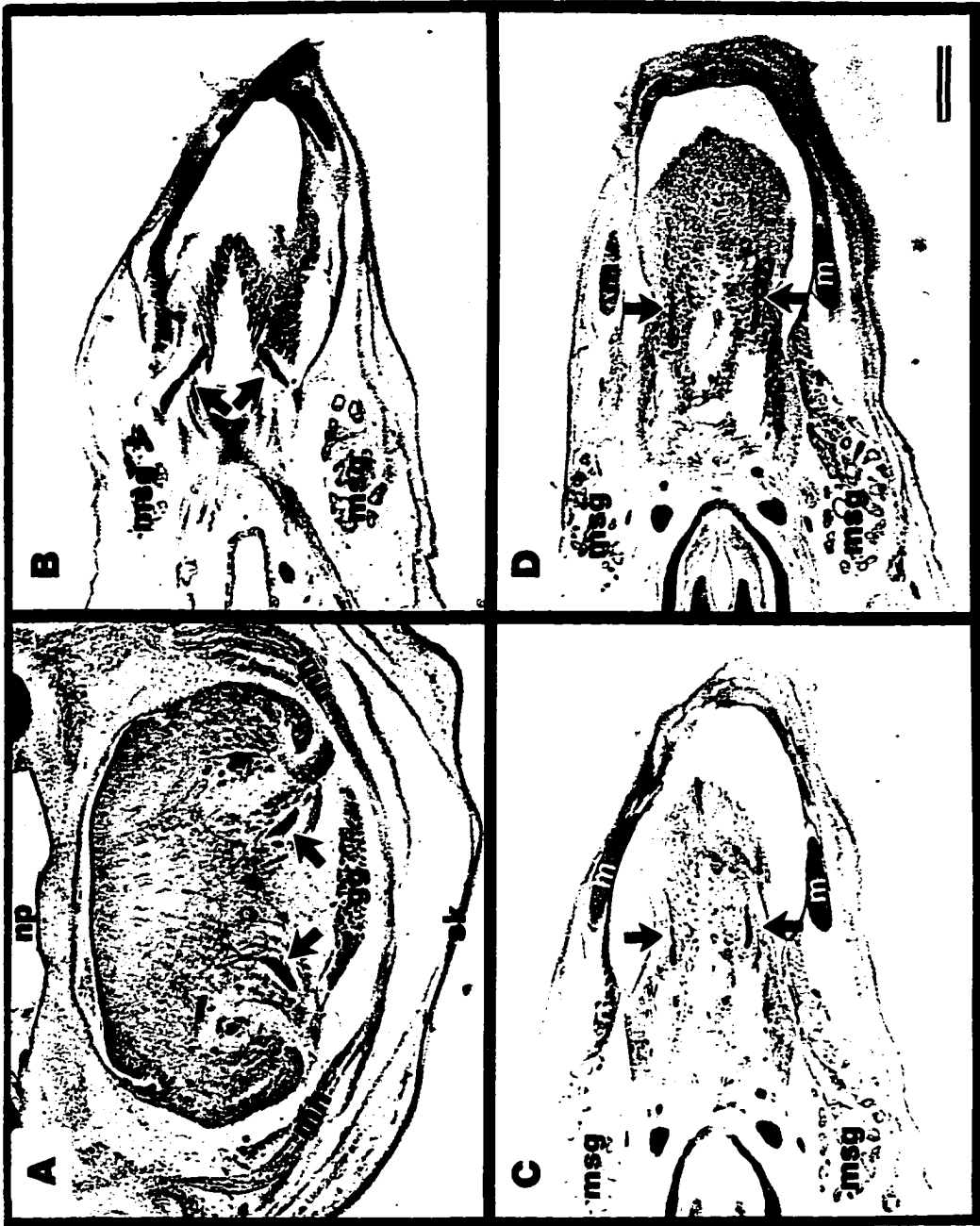


Figure 4.4. A series of photomicrographs demonstrating choline acetyltransferase-like immunoreactivity in efferent projections from hypoglossal motoneurons at 1 PN. Immunoreactivity for choline acetyltransferase was observed in the hypoglossal nerve (arrows): branching (A; coronal section), entering (B; horizontal section), and projecting rostrally (C&D; horizontal sections) in the tongue. Horizontal sections (B, C, & D) are sequential with 40 μm distance inbetween them. Note that the cartilage of the developing skull is dark due to counterstaining of the tissue and does not represent specific immunoreactivity. Abbreviations: gg, genioglossus muscle; m, mandible (Meckel's) cartilage; mh, mylohyoideus muscle; msg, submandibular salivary gland; sk, skin. Scale bars = 200 μm .



hypoglossal nerve can be seen entering the tongue from the periphery (Fig. 4.4B), and extending rostrally through the tongue musculature (Fig. 4.4C,D).

Utilizing whole mount immunohistochemistry for neurofilament one can visualize the hypoglossal nerve at 1 PN (Fig. 4.5). The hypoglossal nerve was observed exiting the brainstem, coursing through the periphery, and branching into the tongue musculature.

Synaptotagmin-like immunoreactivity was observed in the tongue musculature at 1 PN (Fig. 4.6). The hypoglossal nerve labeled with synaptotagmin-like immunoreactivity was localized branching within the tongue musculature (Fig. 4.6A,B). Hypoglossal motoneuron projections contained synaptotagmin-like immunoreactivity and appeared to extend toward and form what appear to be presynaptic terminals onto tongue muscle fibers (Fig. 4.6C,D).

Discussion

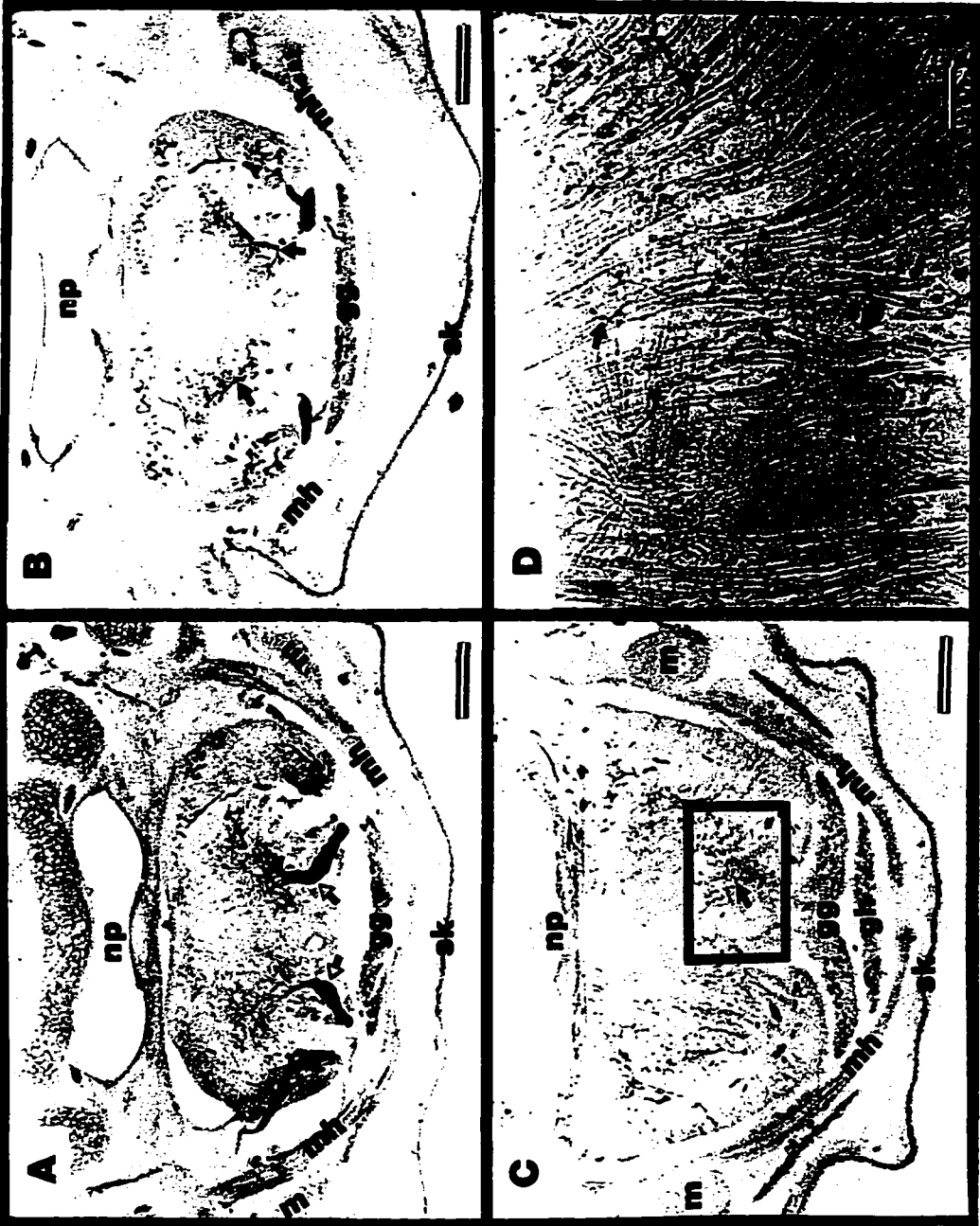
In the present study we have localized the efferent projections from facial and hypoglossal motoneurons in the neonatal Brazilian opossum. Both the facial and hypoglossal nerves project into the periphery at birth. Further, efferent projections from facial and hypoglossal motoneurons were observed to project into the region of target muscles and presumptive developing neuromuscular junctions were observed upon target muscle fibers on the day of birth.

The first component of this study was to examine the developing efferent projections from facial motoneurons that comprise part of the facial cranial nerve. Utilizing choline acetyltransferase-like immunoreactivity we observed projections from facial motoneurons: coursing dorsally toward the genu and then coursing laterally before exiting the rostral medulla. In the periphery the facial nerve was observed exiting the skull and projecting in a rostral direction. Whole mount immunohistochemistry for neurofilament allowed for the visualization of the various branches of the facial nerve at 1 PN. Synaptotagmin-like immunoreactivity was observed within target muscles of the facial motoneurons, indicating that efferent innervation to these muscles might be present.

Figure 4.5. Photomicrographs exhibiting whole mount immunohistochemistry for neurofilament in the 1 PN opossum head. Neurofilament-like immunohistochemistry labels the hypoglossal nerve exiting the brainstem, in the periphery, entering the tongue, and branching through the tongue. Scale bars = 200 μm .



Figure 4.6. A series of photomicrographs, from rostral to caudal (A-C), showing synaptotagmin-like immunoreactivity in hypoglossal motoneuron efferent projections within the tongue at 1 PN. Synaptotagmin-like immunoreactivity reveals the branching of the hypoglossal nerve (A&B) and the appearance of developing neuromuscular junctions upon muscle fibers (C&D). Note that the cartilage of the developing skull is dark due to counterstaining of the tissue and does not represent specific immunoreactivity. Abbreviations: gg, genioglossus muscle, gh, geniohyoidus muscle; m, mandibular (Meckel's) cartilage; mh, mylohyoideus muscle; np, nasopharynx; and sk, skin. Scale bars = 200 μm in A, B, & C, and 50 μm in D.



The second component for this study was to investigate the developing efferent projections from hypoglossal motoneurons that form the hypoglossal cranial nerve. Immunoreactivity for choline acetyltransferase was observed in the hypoglossal nerve at 1 PN. In the periphery the hypoglossal nerve coursed rostrally and medially before entering the tongue musculature and branching throughout this target muscle. Whole mount immunohistochemistry labeled the hypoglossal nerve in the periphery and after it had entered and branched within the tongue musculature. Synaptotagmin-like immunoreactivity was also present within the hypoglossal nerve and developing presumptive presynaptic terminals were observed upon muscle fibers.

These results suggest that both the facial and hypoglossal motoneurons might innervate their respective target muscles on the day of birth (1 PN). The efferent projections were labeled with choline acetyltransferase-like immunoreactivity into the periphery. Whole mount immunohistochemistry was then utilized to label the developing facial and hypoglossal nerves within the peripheral musculature and skin. Finally, synaptotagmin-like immunoreactivity was utilized to look for developing neuromuscular junction terminals upon muscle fibers within respective target muscles.

On the basis of previous studies, hypoglossal motoneurons were expected to innervate their target muscles by birth based upon the importance of the tongue in the suckling mechanisms [26, 51]. However, the idea that facial motoneurons innervate their respective target muscles from birth would not have been predicted. Recent studies in our laboratory, utilizing retrograde tract tracing with cholera toxin subunit B, have demonstrated that the migration of facial motoneurons and the development of the FMN is a postnatal event in the Brazilian opossum [80, 82]. At 1 PN, most facial motoneurons are observed near the developing abducens nucleus. At 3 PN, most facial motoneurons are migrating toward the FMN, and not until 7 PN are almost all facial motoneurons within the FMN. When these previous results are combined with current results presented in this paper it appears that facial

motoneurons are in contact with their target muscles during a time when their cell bodies are migrating to their destination in the facial motor nucleus. This circumstance raises an interesting question - are facial motoneurons functional while migrating? This question is interesting because facial musculature may play a role in suckling behavior.

In addition, studies in our laboratory have focused upon the development of synapses within the FMN in Brazilian opossum neonates. Previously at the light microscopic level we have reported an apparent delayed synaptogenesis within the FMN as compared to that for the hypoglossal motor nucleus (HMN)[81]. Utilizing synaptic terminal-associated markers, we reported that the FMN does not appear to receive afferent innervation until 15 PN, and further, mature synapses are formed between 15 and 25 PN. This delayed synaptogenesis was compared to the HMN which appears to receive afferent innervation from birth. Recently ultrastructure studies of the developing FMN and HMN confirm ongoing synaptogenesis within the FMN but also within the HMN with increasing age [59]. These results again raise the question - are facial motoneurons functional before afferent innervation and if so how are they regulated? We have begun to look for potential regulators and already found that motoneurons in the FMN of the Brazilian opossum transiently express cholecystokinin (CCK) binding sites during this neonatal period [43, 45], although CCK fibers are not detected [23]. We suggest that the activity of facial motoneurons is regulated in a novel or distinct manner compared to hypoglossal motoneurons during this period of brain development.

Summary

In conclusion, immunoreactivity for choline acetyltransferase labels the facial and hypoglossal nerves in the periphery at 1 PN. Whole mount immunohistochemistry for neurofilament demonstrated the developing facial and hypoglossal cranial nerves coursing through the periphery. Synaptotagmin-like immunoreactivity demonstrates the branching of the facial and hypoglossal nerves within their target muscles and the appearance of developing

neuromuscular junctions upon muscle fibers. Taken together these results suggest that both facial and hypoglossal motoneurons have contacted their target muscles from the day of birth during a time when facial motoneurons are still migrating.

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CHAPTER FIVE. GENERAL CONCLUSION

Summary

The first study, described in chapter two, examined the characterization and ontogeny of synapse-associated proteins in the developing facial and hypoglossal motor nuclei in the neonatal Brazilian opossum brain. This study utilized localization of markers associated with synapses to examine their appearance at the light microscopic level. The synapse-associated proteins utilized for immunohistochemistry in this study were: synaptic vesicle-associated proteins, synaptophysin and synaptotagmin; a synaptic membrane protein, plasma membrane-associated protein of 25 kDa (SNAP-25); a growth cone protein, growth-associated phosphoprotein-43 (GAP-43); and microtubule-associated proteins, axonal marker Tau and dendritic marker microtubule-associated protein-2 (MAP-2). Results from this study demonstrated that during the first ten postnatal days (1-10 PN), the FMN lacked immunoreactivity for synaptophysin, synaptotagmin, GAP-43, Tau, and SNAP-25. After 10 PN, immunoreactivity increased in the FMN for synaptophysin, synaptotagmin, GAP-43, and Tau, whereas immunoreactivity for SNAP-25 was not evident until between 15 and 25 PN. Conversely, immunoreactivity for MAP-2 was present in the FMN from 5 PN onward. In contrast to the FMN, the HMN displayed immunoreactivity from 1 PN for synaptophysin, synaptotagmin, SNAP-25, GAP-43, Tau, and MAP-2. These results suggest that the FMN of the Brazilian opossum may not receive afferent innervation as defined by classical synaptic markers until 15 PN, and further, that characteristic mature synapses are not present until between 15 and 25 PN. These results indicate that there may be a delay in synaptogenesis in the FMN as compared to synaptogenetic events in the HMN.

The morphological development of the facial and hypoglossal motor nuclei in the neonatal Brazilian opossum brain was discussed in chapter three. In this study, we utilized retrograde tract tracing through the postnatal injection of cholera toxin subunit B (CtB) to characterize the formation of the facial and hypoglossal motor nuclei in the developing neonatal

opossum brainstem. Injections of CtB were made into the cheek / lip region or tongue of opossum pups to retrogradely label the facial or hypoglossal motor nuclei, respectively. Facial motoneurons in newborn opossum pups (1 PN) exhibited CtB labeling following a two hour survival time, with their cell bodies essentially localized near the developing cranial abducens nucleus. At 3 and 5 PN, following a forty-eight hour survival time, CtB labeled facial motoneurons were observed in and migrating to the region of the adult facial motor nucleus in the rostral medulla. Between 7 and 10 PN, almost all facial motoneurons had migrated to their destination within the facial motor nucleus. Hypoglossal motoneurons also exhibited CtB labeling from 1 PN, however, their cell bodies were localized within the hypoglossal motor nucleus at the earliest age examined. Double label studies, to examine guidance of facial motoneurons during migration, demonstrated that CtB labeled facial motoneurons are in close proximity to vimentin-like immunostained radial glial fibers during migration. These results suggest: 1) the migration of facial motoneurons to the facial motor nucleus is a postnatal event, 2) that efferent projections from facial and hypoglossal motoneurons project into the peripheral region of their target muscles from the day of birth, and 3) facial motoneurons migrate to their destination in the brainstem thereafter, in close association with radial glial fibers.

The third study, presented in chapter four, examined the efferent projections of facial and hypoglossal motoneuron projections in the neonatal Brazilian opossum. To localize the efferent projections from facial and hypoglossal motoneurons we utilized immunohistochemical detection of choline acetyltransferase, neurofilament, and synaptotagmin. Facial and hypoglossal motoneurons and nerves demonstrated choline acetyltransferase-like immunoreactivity (ChAT-IR) from the day of birth (1 PN). At 1 PN, the facial nerve, labeled with ChAT, was observed exiting the skull, extending rostrally, and branching in its target muscles. In addition, the caudal auricular, temporal, zygomatic, buccal, and marginal mandibular branches of the facial nerve were observed utilizing immunohistochemistry for neurofilament in whole mount preparations. The hypoglossal nerve, labeled at 1 PN with

ChAT-IR, was also observed in the periphery extending toward, entering into, and branching within the tongue. In whole mount preparations at 1 PN, the hypoglossal cranial nerve was again observed exiting the skull, coursing through the periphery, and entering into the tongue. Further, at 1 PN, presynaptic terminals were localized in target muscles innervated by either facial or hypoglossal motoneurons, using immunohistochemistry for synaptotagmin-like immunoreactivity. Facial motoneurons in the Brazilian opossum are actively migrating for the first postnatal week. However, these data suggest that facial and hypoglossal motoneurons have contacted, and may innervate their target muscles from the day of birth, a time during which facial motoneurons are still migrating.

Conclusions

Various components of facial and hypoglossal motor systems development were examined in the neonatal Brazilian opossum brain in these studies. Specifically, we examined the time course of afferent innervation, migration and nuclear development, and establishment of efferent innervation. From these studies we have obtained results that can be expounded in the following areas:

Afferent Innervation to the FMN and HMN

Afferent innervation to the FMN has been investigated in the adult rat and North American opossum (Dom et al., 1973; Travers, 1995; Travers and Norgren, 1983). In addition, afferent innervation to the HMN has been reported in the adult rat (Borke et al., 1983). The ontogeny of afferent innervation to either the FMN or HMN has not been previously examined in any species. However, in the rat, the peptidergic and aminergic innervation to the FMN was reported to increase during the first week of postnatal life, with an established innervation pattern appearing by 10 PN (Senba et al., 1985). The first study, presented in chapter two, examined the appearance of synapse-associated proteins in the neonatal Brazilian opossum FMN at the light microscopic level. Localization of synapse-associated proteins were utilized to provide insight into the time course of afferent innervation

to the FMN and HMN.

Results from this study suggest that there is a delay in the appearance of synapse-associated markers in the FMN as compared to the HMN. Levels of immunostaining for synapse-associated proteins were lower in the FMN from birth until 10 PN and increased throughout the period investigated. Conversely, immunostaining for synapse-associated proteins were detected in the HMN from the day of birth. We interpret these results to indicate that afferent innervation to the FMN is delayed when compared to that for the HMN. Further, our findings indicate that the FMN, in contrast to the HMN, has decreased levels of synaptic or “classical” innervation during this period of postnatal brain morphogenesis.

These results for the FMN are somewhat similar to peptidergic and aminergic innervation study by Senba and coworkers indicating that the neonatal rat FMN does not receive specific types of innervation until after 10 PN (Senba et al., 1985). Our study suggest that the FMN of neonatal Brazilian opossums does not receive afferent innervation until after 10 to 15 PN as well (Swanson et al., 1996). More studies are needed in other species but from these two studies their results suggest that maybe delayed afferent innervation to the FMN might be a common trend among species.

Additional studies in our laboratory have investigated synaptogenesis within the FMN and HMN of the Brazilian opossum at the ultrastructural level (Pepper et al., 1996). Results from this study indicate that synaptogenesis within the FMN is ongoing during the postnatal period with the number of synapses increasing 3.5 times between 5 and 25 PN. In addition, this study demonstrated that synaptogenesis within the HMN is also ongoing during the postnatal period. From this study we concluded that synaptogenesis is ongoing within both the FMN and HMN during the first two postnatal weeks in the Brazilian opossum neonate.

To explain the discrepancy between the light microscopic study in chapter two and electron microscopic study we believe that the difference lies between the two techniques. The electron microscopic study examined the appearance of synapses at the anatomical /

ultrastructural level within the two nuclei. In contrast, the light microscopic study examined the appearance of immunohistochemistry against synapse-associated proteins within the two nuclei. While the results of the electron microscopic study indicate the true development of synaptogenesis within the two nuclei, the results from the light microscopic study might imply the development of functional synapses within the two nuclei. For example, current models for regulated vesicle fusion and exocytosis in eukaryotic cells include both synaptotagmin and SNAP-25 as integral components during synaptic vesicle exocytosis (Bark and Wilson, 1994; O'Connor et al., 1994). While the absence of immunoreactivity cannot exclude synapses from the FMN, two studies demonstrate that synapses devoid of immunoreactivity would have decreased function. Mutants with a deficiency for synaptotagmin are lacking excitation produced secretion at the synapse (Broadie et al., 1994; Geppert et al., 1994). Synapses are present in these mutants but only spontaneous activity exists at those synapses.

Development of the FMN and HMN

Development of the FMN and HMN was investigated in the study presented in chapter three. In placental mammals the migration and development of the FMN and HMN are prenatal events that occur *in utero*. In the Brazilian opossum the development of the HMN appears to be a prenatal event. On the day of birth (1 PN) the retrogradely labeled hypoglossal motoneurons were localized within the HMN. In contrast, results presented in the chapter three study demonstrate the postnatal FMN development and facial motoneuron migration. On the day of birth (1 PN) retrogradely labeled facial motoneurons were essentially localized near the developing abducens nucleus. At 3 and 5 PN, facial motoneurons were localized in-between the abducens nucleus and the developing FMN. Between 7 and 10 PN almost all facial motoneurons have migrated to their destination within the FMN. This postnatal development of the FMN was not expected considering that facial motoneurons might be necessary for the function of facial musculature during this postnatal period.

Migration of facial motoneurons

As mentioned, the migration of facial motoneurons appears to be a postnatal event in Brazilian opossum neonates. During the migration of facial motoneurons they have a characteristic bipolar shape with a leading and a trailing process. As previously described for other migrating neurons, the classical description of a migrating neuron or motoneuron is a bipolar shape with leading and trailing processes oriented in the direction of the migration (Levi-Montalcini, 1964). However, in this situation for facial motoneurons the trailing process is the efferent axon projecting to the respective target muscles.

In addition to displaying the characteristics of a migrating neuroblast, our results indicate that additional factors indicate that facial motoneurons are migrating. The rate of facial motoneuron movement is different than the rate of brainstem expansion (see Table 3.2). From 1 to 7 PN facial motoneuron displacement occurs at an average rate of 87.5 microns per day. During the same period the brainstem expands in height at an average rate of 65.0 microns per day. Thus, the facial motoneurons are traveling in the vertical direction around an average of 20+ microns per day more than the brainstem is expanding. However, the movement of facial motoneurons in the horizontal direction away from the midline occurs at 48.7 microns per day while the brainstem is expanding in width at an average rate of 94.3 microns per day. Since the brainstem width expansion is twice the distance from the midline the adjusted expansion rate for one half of the brainstem would be 47.2 microns per day, which is very similar to the rate of facial motoneuron movement. From these data we conclude that facial motoneurons do migrate due to their faster movement in the vertical direction than brainstem height expansion. However, the question of facial motoneuron migration in the horizontal direction is unanswered since it is at a similar rate to the expansion of the brainstem in width. Further statistical analysis will be performed to determine the significance of these rates.

Further, CtB retrogradely labeled facial motoneurons were localized in close proximity to vimentin-like immunostained radial glial fibers. Previous studies have demonstrated some

of the associations of radial glial fibers with migrating neuroblasts. It is believed that radial glial cells act as physical scaffolds and substrates along which neurons can migrate (reviewed in Rakic, 1988; Rakic, 1990; Rakic, 1991). These studies have observed close associations between migrating neuroblasts and radial glial cells in the developing cerebral cortex, cerebellum, and tectum (Goldowitz and Mullen, 1982; Rakic, 1971; Rakic, 1972; Vanselow et al., 1989). In chapter three we have identified close associations between retrogradely labeled facial motoneurons and immunostained radial glial cells. The radial glial fibers are aligned in a vertical orientation for guidance of facial motoneurons during their migration from their site of neurogenesis to their final location within the FMN. We believe that radial glial fibers may provide facial motoneurons support and guidance during their migration.

These data confirm that facial motoneurons are migrating postnatally. The results demonstrate that facial motoneurons have the characteristic shape of migrating neuroblasts, their rate of movement is faster than the expansion of the brainstem, and they are in close association with radial glial fibers previously demonstrated to guide migrating neurons.

Efferent Innervation from the FMN and HMN

From the day of birth both facial and hypoglossal motoneurons appear to innervate their respective target muscles. The study presented in chapter four examined the anatomical distribution of facial and hypoglossal motoneuron projections on the day of birth. Innervation of the tongue musculature by the hypoglossal motoneurons would be expected considering their importance in the suckling mechanisms. Studies have reported that mammals utilize their tongue musculature in a pump-suck mechanism that requires tongue movements to create a vacuum for milk expulsion (German and Crompton, 1996; Müller, 1968). However, innervation of the facial musculature by facial motoneurons from the day of birth was not as predictable. Most researchers consider the suckling mechanism to mainly utilize the tongue musculature, but our laboratory believes that the facial musculature is necessary for suckling behaviors in addition to the tongue musculature. Innervation of facial musculature by facial

motoneurons during this period suggests that those muscles are capable of contributing to oromotor function and could be involved in suckling behavior.

General Conclusions

The three studies presented in this dissertation illustrate an interesting developmental situation for facial motoneurons. From the third study we determined that facial motoneurons have efferent projections that extend into the periphery and to the respective target muscles at birth. This process of facial motoneurons innervating their target muscles occurs during a time when their cell bodies are migrating through the brainstem, as shown in study two. From the first study we know that the time course of synapse-associated protein appearance is delayed in the FMN as compared to the HMN. This apparent delay of afferent innervation to the FMN during the first five days can be explained because the facial motoneurons are not there. However, from 5 to 10 PN, when most of the facial motoneurons are located within the FMN, afferent innervation appears to be absent.

From this scenario of FMN development in the Brazilian opossum during the first two postnatal weeks several questions arise. First, are the facial motoneurons functional during this time period? The facial motoneurons are connected to their target muscles and are presumably capable of innervating these muscles. If facial motoneurons are capable of innervating their target muscles they would do so while their cell bodies are in the process of migrating. A motoneuron or neuron innervating their target during migration is an unheard-of occurrence during brain morphogenesis and central nervous system development. Second, if facial motoneurons innervate their target muscles during this time period, how would they be regulated? The first study demonstrated that the appearance of synapse-associated proteins is delayed for the first ten to fifteen days. This question of facial motoneuron regulation could be applied to either during their migration to the FMN or after they are located within the FMN during this time period. Our laboratory believes that facial motoneuron regulation is not through "classical" afferent sources but rather through transiently expressed receptors.

Studies by several laboratories demonstrate that facial motoneurons transiently express a number of compounds and receptors. During a similar time course to studies presented in this dissertation, facial motoneurons transiently express: vasopressin binding sites in the embryonic and neonatal rat (Tribollet et al., 1991), cholecystinin binding sites in the neonatal Brazilian opossum (Kuehl-Kovarik et al., 1993a), cholecystinin binding sites in the embryonic and neonatal rat (Kuehl-Kovarik and Jacobson, 1996), estrogen receptors in the neonatal rat (Yokosuka and Hayashi, 1992), nerve growth factor (NGF) receptors in the embryonic and neonatal rat (Friedman et al., 1991; Yan and Johnson, 1988), and the enzyme adenosine deaminase (ADA) in the embryonic and neonatal rat (Senba et al., 1987). The significance of these receptors and compounds being expressed transiently is unclear. These transiently expressed receptors could have a physiological role in facial motoneuron regulation during a period of decreased or absent afferent innervation.

The role of transient binding sites is confusing. Binding sites for CCK were observed transiently in the neonatal Brazilian opossum (Kuehl-Kovarik et al., 1993a) and rat (Kuehl-Kovarik and Jacobson, 1996) FMN and vasopressin binding sites were observed in the neonatal rat FMN (Tribollet et al., 1991). Yet studies from our laboratory have demonstrated that neither CCK nor AVP immunoreactive fibers are found in this brainstem region (Fox et al., 1990; Iqbal and Jacobson, 1995a). This circumstance of binding sites without appropriate peptide terminals is known as a mismatch (Herkenham, 1987). The mismatch phenomenon is common throughout the central nervous system (reviewed in Herkenham, 1987) and occurs in several systems such as circadian rhythms (Rusak and Bina, 1990) and respiratory control (Moss et al., 1986). In this mismatch situation the information flow is thought to occur in a "parasympathetic" manner with transmitter action from a distance (reviewed in Herkenham, 1987).

Synaptogenesis within the FMN appears following a time when facial muscle movement takes place. Thus this delay in maturation of the FMN circuitry may allow for an additional layer of plasticity in the regulation of the neuromuscular control of food intake.

Further, these results suggest that function / activity of facial motoneurons is regulated in a novel or distinct manner compared to that of hypoglossal motoneurons during brain development.

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BIOGRAPHICAL SKETCH

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