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An electron microscopic study of developing facial and hypoglossal motor nuclei in the Brazilian opossum

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CHAPTER 1. INTRODUCTION

The development of the mammalian brain is extremely complex. Although well studied, researchers have little understanding of the dynamics of central nervous system development. Many different animals are utilized for experimentation with the brain including eutherian mammals such as the rat, the cat, the dog, the mouse, and the monkey. In addition, multiple marsupial species, and also various non-mammalian species have been studied. Many of these species are limited in their accessibility when studying neuronal development. The marsupial however is an excellent animal for studying the developing central nervous system.

Monodelphis domestica

The Brazilian opossum, *Monodelphis domestica*, is a small (adult body weight 70-120 grams), rodent-like, pouchless marsupial from southeastern South America (Saunders et al., 1989). The young are extremely immature at birth; many organs are at an embryonic stage of development (Saunders et al., 1989). Researchers began studying the central nervous system of this marsupial in the late 1980's and have found it to be a useful, manageable tool. It breeds well in captivity bearing litters of up to 13 pups (Fadem et al., 1982). Utilizing *Monodelphis* for experimentation makes accessible "fetus-like" pups without the complications of cesarean section as would commonly be the case when working with eutherian mammals.

Being a marsupial, *Monodelphis* pups are born in an extremely undeveloped state and must locate and attach to a teat if they are to survive. By the first day of birth (1 day of age postnatal = 1PN), these pups have latched onto a teat and will remain physically attached for two weeks by a membrane-like epithelium called the epitrichium. Apparently, the epitrichium

connects at the corners of the pup's mouth which helps to form a tube around the mother's teat (Hughes and Hall, 1988). When the teat swells, the pup remains attached. Although the epitrichium atrophies after two weeks, the neonates suckle at will until approximately 40PN when they begin to eat solid food (Fadem et al., 1982). The neonatal pups are readily accessible on the underbelly of the mother. In addition, studies utilizing *Monodelphis* pups can include littermates of various ages since not all pups need to be removed at one time. This would not be possible in the case of cesarean removal of fetuses at similar stages of development in eutherian mammals.

Monodelphis is an excellent laboratory animal to study development of the central nervous system (CNS) because of its unique marsupial attributes, and small size (as opposed to *Didelphis* or other larger marsupial species) allowing for ease in handling and experimental manipulations. *Monodelphis* is equipped for survival with various systems well developed at birth. One specific system of interest is the oromotor system involved in suckling behavior. The mechanisms involved in the sucking response suggest a greater amount of development is required in the oromotor system of *Monodelphis* compared to other eutherian mammals at similar stages (about embryonic day 13 in the rat; Saunders et al., 1989). The unique attributes of this animal's well developed systems for survival poses a fascinating question: If the young are born in such an undeveloped state within the CNS, what neural connections are present allowing for the oromotor system to function? There must be some regulation of the systems involved in the suckling mechanism in the developing neonate.

CNS Development

It has previously been shown that neuronal proliferation (growth) in the forebrain of *Monodelphis* is not exclusively a prenatal process. In fact, many forebrain structures do not complete neurogenesis until well after the birth of the animal (Iqbal et al., 1995; Larsen and Jacobson, 1986; Saunders et al., 1989; Swanson et al., 1994; Rivkees et al., 1988). Postnatal neurogenesis is also evident in portions of the cerebral cortex, in the midbrain colliculi, and in the cerebellum. Therefore, *Monodelphis* potentially regulates (without forebrain involvement) homeostatic processes via regulatory centers already present in the brain (most likely the brainstem) at birth. These centers, called nuclei, contain the cell bodies of neurons that either make connections (synapse) with other areas within the brain or extend their axons, exiting the brainstem, to form specific sensory, parasympathetic, and motor pathways. These could include the oromotor nuclei involved in the suckling behavior, all of which are located in the midbrain or hindbrain. There are three specific motor nuclei involved in oromotor function: the trigeminal motor nucleus (TMN), the facial motor nucleus (FMN), and the hypoglossal motor nucleus (HMN). The TMN is specifically involved in the innervation of muscles associated with chewing and mastication and, therefore, has not been examined in this study. The neurons of the HMN solely innervate muscles of the tongue and the neurons of the FMN innervate peripheral muscles of the face. For this reason, the HMN and FMN are hypothesized to be involved in suckling behavior. Further studies have looked specifically at the FMN and HMN and their development.

There seems to be a difference in the development of the FMN when compared to the HMN. Studies considering the ontogeny of these oromotor nuclei have shown a delayed

organization of the FMN (Swanson et al., 1996). These motor nuclei contain large motoneurons and surrounding neuropil. The neuropil contains support cells, vasculature, axonal fibers, dendrite processes and their associated synapses. When looking at the development of the HMN, their large motoneuron cell bodies are localized in the nucleus at 1PN whereas other motoneurons are migrating to the FMN from 1PN to 5PN (Swanson et al., 1996). These nuclei also have been examined for the expression of specific proteins associated with synapses and synaptic vesicles, as well as process extensions utilizing immunohistochemical markers. The specific immunohistochemical markers include the synaptic vesicle-associated proteins synaptophysin and synaptotagmin; a synaptic membrane protein, plasma membrane-associated protein of 25kDa (SNAP-25); a growth cone protein, growth-associated phosphoprotein-43 (GAP-43); and the microtubule-associated proteins axonal marker (τ) and dendritic marker microtubule-associated protein-2 (MAP-2; Swanson et al., 1996). Synaptic formation has been reported to correlate with the expression of synaptophysin (p38; Leclerc et al., 1989; Laemle et al., 1991; Masliah et al., 1991), synaptotagmin (p65; Lou and Bixby, 1993), synaptosomal-associated protein of 25 kDa (SNAP-25; Catsicas et al., 1991; Bark, 1993), 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 parts of the neuron (Swanson et al., 1996). This study found that during the first 10 postnatal days (1-10PN), the FMN lacked immunoreactivity for synaptophysin, synaptotagmin, GAP-43, τ, and SNAP-25. Conversely, immunoreactivity for MAP-2 was present in the FMN at birth. In

contrast, the HMN displayed immunoreactivity from 1PN for synaptophysin, synaptotagmin, SNAP-25, GAP-43, τ, and MAP-2 (Swanson et al., 1996).

These results suggested that the FMN of *Monodelphis* may not receive afferent innervation as defined by classical synaptic markers until 15PN. Furthermore, characteristic mature synapses are not present until between 15 and 25PN (Swanson et al., 1996). This investigation led to an interest in examining these two oromotor nuclei at the electron microscopic level to see if the difference in the expression of the proteins could be anatomically related to the appearance of synapses. Is there an apparent delay in synaptogenesis in the FMN compared to the HMN? Is a significant portion of synaptogenesis in the opossum FMN postnatal?

Thesis Organization

The CNS of *Monodelphis domestica* is a unique system for studying neuronal development. There is very little forebrain development, yet at birth the animal still manages to utilize specific oromotor systems involved in suckling behavior. The purpose of this study was to specifically examine the FMN and the HMN at the electron microscopic level, and to explore synaptic development in these oromotor nuclei in the Brazilian opossum. This study has demonstrated, utilizing electron microscopic techniques, an apparent development of synapses with age in both the FMN and HMN. This thesis describes the background research that supported the project and explains the techniques involved in collecting the data at the ultrastructural level. The results are presented accompanied by a discussion explaining why the data supported the theory that a substantial amount of development is ongoing in the neonatal Brazilian opossum.

CHAPTER 2. LITERATURE REVIEW

The neonatal marsupial is equipped with some well-developed systems for survival, especially those associated with oromotor function. The skull's development, including both the onset of ossification and the rate of bone growth, around the oral cavity is accelerated (Clark and Smith, 1993). This makes the opossum more adaptive to suckling. One theory as to why there is such development in the oral area is based on the possible 'pump-sucking' mechanism utilized by the suckling neonate (German and Crompton, 1996). This mechanism involves pushing the tongue against the teat toward the roof of the mouth and then pulling the tongue away from the teat creating negative pressure for milk expulsion. This theory implies a potentially well-developed motor system for muscles in the tongue (for pumping and pressure) as well as for facial muscles involved in sucking. The two oromotor pathways that could potentially be involved are the facial motor and the hypoglossal motor systems.

The muscles of the tongue are innervated by efferent fibers extending from cell bodies located in the hypoglossal motor nucleus (HMN). These fibers extend out of the brainstem and make up a portion of the hypoglossal nerve (12; Evans and Christensen, 1979). The facial motoneuron cell bodies reside in the facial motor nucleus (FMN) and their efferent fibers extend out of the brainstem and form part of the facial nerve (7; Evans and Christensen, 1979). Both of these motor nuclei are innervated by afferent projections from many different centers in the brain. There is no evidence of any direct afferent projections to either the facial or hypoglossal from centers in the forebrain (Travers, 1995). Indirect projections pass through the reticular formation from the motor cortex. Direct afferent projections come from the midbrain, and hind brain. The innervation of these two motor nuclei, specifically the

presence of synapses, suggests that the connections are there for normal functional movement of the muscles which the motoneurons innervate.

In order to better understand developing synaptogenesis in the FMN and HMN it is important to examine where these nuclei are located within the mammalian brain, as well as the afferent innervation from sensory and motor nuclei involved in these oromotor systems. The FMN and HMN are located in different parts of the medullary brainstem and are regulated by and receive afferents from both similar and distinct areas. It is also important to know how these nuclei appear in neonatal *Monodelphis* and how other mammalian brain nuclei have been studied, at the level of the synapse, during development.

Facial Motor Nucleus

The mammalian facial motor nucleus (FMN) contains somatic motor neurons of the facial nerve ranging in size from 7-60 μm in diameter (Dom et al., 1975). In the rat, the FMN is located in the rostral medulla ventromedial to the spinal nucleus of the trigeminal nerve (Paxinos et al., 1994). In the dog and the rat, the nerve fibers course dorsorostrally extending to the floor of the fourth ventricle dorsal to the abducens nucleus (Evans and Christensen, 1979). The fibers curve around the abducens nucleus forming the facial genu. They then course laterally between the dorsal nucleus of the trapezoid body and spinal nucleus of the trigeminal, and exit the medulla immediately ventral to the vestibulochoclear nerve (Evans and Christensen, 1979). In *Monodelphis*, the nerve courses along a similar path until after the genu where the nerve exits laterally without turning ventrally as in the dog and rat (Swanson, personal communication). Somatic efferents originating in the rat FMN distribute to the muscles of facial expression for example, the anterior auricular, posterior auricular, orbic

oculi, nasolabialis, platysma, dilator naris, zygomaticus and posterior belly of the digastric (Watson et al., 1982). Cells within the FMN are segregated into several subdivisions consisting largely of groups of cells that are separated by white matter, but not by obvious cytoarchitectonic differences. These subdivisions are distinctly different between species. In the rat, lateral, dorsolateral, intermediate, and medial subdivisions in the FMN are readily apparent in most Nissl-stained coronal sections (Travers, 1995). These subdivisions however are not apparent in *Monodelphis*. The topographic organization of the FMN in the rat reflects the cells of origin for specific facial nerve branches as well as the representation of individual facial muscles. In the rat, a small amount of neurons project from the FMN to the cerebellar flocculus and not to the facial musculature (Travers, 1995).

Different subdivisions of the FMN of the rat receive substantial input from centers of the midbrain, pons, and medulla. In the midbrain, cells anterior to the contralateral nucleus of the lateral lemniscus project specifically to the medial subdivision of the FMN. In the cat, this region of the midbrain receives input from the superior colliculus and has been implicated in pinna orientation to sound (Travers, 1995). Scattered cells in the central gray, periocular nuclei, olivary pretectal nucleus, and the midbrain reticular formation also project to the FMN of the rat and provide a pathway for facial movements associated with emotional behaviors, reflexes of the eye, and vocal-facial behavior. The lateral and intermediate subdivisions of the rat FMN receive projections from the contralateral red nucleus and provide a relay for cortical and cerebellar input to the FMN (Travers, 1995).

In the pons of the rat, cells of the ipsilateral Kolliker-Fuse nucleus and the supratrigeminal and ventrolateral parabrachial regions project to the lateral and intermediate

divisions of the FMN. The medial parabrachial nucleus may be involved in exploratory snuffing, nasal breathing, and vibrissae movements, activities requiring coordination between respiratory centers and facial motoneurons. Likewise, the ventral parabrachial region could mediate gustatory evoked facial responses (Travers, 1995).

In the rat medulla, most of the projections to the FMN originate from cells in the reticular formation (Travers, 1995). The reticular formation exerts powerful excitatory and inhibitory influences over a wide variety of motor activity (Evans and Christensen, 1979). Different regions of the rat medulla project to different divisions of the FMN, although these differences are not as pronounced from the midbrain and pons. At the level of the rat hypoglossal motor nucleus, many reticular cells project to the intermediate and lateral divisions of the FMN. Cells in the caudal medulla that project to the medial subdivision of the rat FMN tend to be ventrally located and include cells in and around the nucleus ambiguus (Travers, 1995). Other medullary projections to the rat FMN originate from the spinal nucleus of the trigeminal and go to the medial subdivision of the FMN (Travers, 1995).

Hypoglossal Motor Nucleus

The hypoglossal motor nucleus (HMN) of the rat, dog, and *Monodelphis* is in the medulla adjacent to the midline and floor of the fourth ventricle (Paxinos et al., 1994). The motoneurons of the rat HMN are arranged in a few groups, each of which innervates a separate tongue muscle (Travers, 1995). The axons from the hypoglossal nucleus extend in the ventrolateral direction, cross the olivary nucleus and emerge at the medulla immediately lateral to the pyramid. The nerve roots then merge to form the hypoglossal nerve (Altman and Bayer, 1995). The tongue consists of both extrinsic (genioglossus, styloglossus, hyoglossus,

and palatoglossus) and intrinsic (vertical, transverse, superior longitudinal, and inferior longitudinal) musculature. Most of the motoneurons innervating the lingual musculature originate from the HMN and travel in the hypoglossal nerve. The myotopic organization of the rat HMN reflects three anatomical and functional relationships. First, protrudor and retractor motoneurons are organized dorsoventrally with retractor motoneurons located dorsally and protrudor motoneurons ventrally. Second, motoneurons innervating the intrinsic musculature are generally medial to those innervating the extrinsic musculature. Third, peripheral topography is maintained such that motoneurons innervating the most distal muscles or muscle fibers are located most caudally. Each rat HMN contains approximately 3,500 neurons. Most of the cells within the HMN of the rat are motoneurons but there is anatomical evidence for a small population of interneurons (Travers, 1995). At the ultrastructural level, small interneurons have invaginated nuclei and little cytoplasm in contrast to large motoneurons that contain large centrally placed round nuclei and abundant cytoplasm (Travers, 1995).

Afferent projections reach the HMN of the rat from cells in the midbrain, pons, and medulla. These projections originate both from well-defined cell groups in identified brain stem nuclei and from more widely distributed, poorly defined regions of the reticular formation. In the midbrain, a few cells in the contralateral reticular formation project to the HMN. In a study on the opossum, localized midbrain projections came primarily from the central gray matter. This suggests that the mastication muscles and lingual muscles are influenced by proprioceptive stimuli (Travers, 1995). In the pons, neurons that project to the HMN are from the medial parabrachial and intertrigeminal nuclei, Kolliker-Fuse nucleus, and

the subcoeruleus nucleus. A primarily ipsilateral projection from the trigeminal sensory nucleus reaches the HMN from a distribution of relatively large cells located dorsomedially in the caudal two thirds of the nucleus (Travers, 1995). In the medulla, the majority of cells projecting to the HMN originate from the medullary reticular formation. Another distribution of cells projecting to the HMN originate in the dorsal part of the spinal trigeminal nucleus pars oralis and pars interpolaris. In the cat, these neurons are associated with polysynaptic trigemino-lingual reflexes elicited from electrical stimulation of the trigeminal nerve. Relatively few cells project from the solitary nucleus to the HMN (Travers, 1995).

Both the FMN and HMN receive input from various regions in the midbrain, pons and medulla. No research has been done yet on the development of afferent innervation to either nucleus in *Monodelphis* as to when specific afferents reach these nuclei. Some work has been done in the rat as to discriminate ultrastructurally the types of synapses associated with specific afferents to motoneurons of the hypoglossal from trigeminal and reticular projections (Borke and Nau, 1985; Borke and Nau, 1987; Borke et al., 1988).

FMN and HMN in *Monodelphis*

In *Monodelphis*, recent studies were conducted on the characterization and ontogeny of developing facial and hypoglossal nuclei. Examination of the time course of migration of facial and hypoglossal motoneurons as well as their nuclear organization has demonstrated a difference in their development (Swanson et al., 1996). The large motoneurons of the HMN were present by 1PN whereas facial motoneurons migrated to their final destination between 1 and 10PN. Visualization of this phenomenon was done by utilizing postnatal injections of the retrograde tract tracer Cholera toxin subunit B and immunohistochemical labeling (Swanson

et al., 1996). By 5PN most of the facial motoneurons had reached the FMN and by 10PN they all had migrated to their target. Immunohistochemical studies indicated a delayed synaptogenesis occurring in the FMN when compared to the HMN.

Synapse Identification

It is relatively simple to identify synapses in neural tissue due to the high affinity the pre- and post-synaptic membranes have to the heavy metal counterstains (osmium tetroxide, uranyl acetate, and lead citrate) used to enhance contrast in the thin sections made for electron microscopic evaluation. Ultrastructurally, synapses occur between axons and cell bodies, axons and dendrites (proximal, intermediate and distal) and between axons. The synaptic junction consists of the pre- and post-synaptic membranes, the densities associated with their cytoplasmic faces, and the synaptic cleft between them (Peters et al., 1976). Synapses are continuously changing size, shape, and position in response to many diverse events. Electron densities associated with post-synaptic cell membranes are composed of globular proteins resting on a filamentous cytoskeleton. One of the major globular proteins may regulate the turnover of the postsynaptic cytoskeleton and thus contribute to synaptic plasticity (Cross and Mercer, 1993). Previous studies in the rat have indicated a plastic effect in some synapse formation, increasing in length and density with age (Aghajanian and Bloom, 1967; Markus et al., 1987). Other methods for visualization of the synaptic profiles were used in these studies, specifically the use of phosphotungstic acid in a technique to label only synaptic profiles at the electron microscopic level. These studies were performed on perfused rats and, therefore, obtained good morphology at the ultrastructural level (Aghajanian and Bloom, 1967).

Previous studies on the cytology and synaptic organization in the FMN of the adult opossum *Didelphis virginiana* have shown an appearance of three types of neurons present in the FMN, specifically, large neurons (50-30 μm), medium neurons (30-20 μm), and small neurons (20-10 μm; Falls and King, 1976). Pre-synaptic profiles on the cell bodies of large, medium, and small facial neurons were grouped into three primary categories based on differences in vesicle shape; spherical, pleomorphic, or flattened. These results provided evidence for a considerable range in the size of the synaptic vesicles within each of the three types. These different vesicle populations may reflect any of the potential differences in axon systems, or taken together with the other structural features, they could represent distinctive axon systems that synapse within the FMN (Falls and King, 1976).

Synapse Development

Developmental synaptogenesis has previously been demonstrated in the rat by utilizing electron microscopy and immunoreactivity for different synapse associated proteins in various brain nuclei. Synaptogenesis was studied in the rat suprachiasmatic nucleus using quantitative ultrastructural analysis and synapsin I immunoreactivity (Moore and Bernstein, 1989). The methodology for examining the synapses at the ultrastructural level included utilizing micrographs non-systematically taken to include perikera, blood vessels and axon bundles. The counts, therefore, represented numbers from the suprachiasmatic nucleus and not just neuropil. The total area evaluated per sample was 1000 μm^2. A synaptic profile was counted if there was an evident membrane thickening and three or more synaptic vesicles nearby (Moore and Bernstein, 1989). The quantitative analysis of synaptogenesis in the rat illustrated very rapid postnatal synaptogenesis for the whole suprachiasmatic nucleus between 3PN and

10PN. Other studies of synaptogenesis in olfactory centers in developing rats utilized similar ultrastructural techniques. The middle portion of the olfactory bulb was dissected out and processed for electron microscopy. Sections were evaluated and counts of synaptic profiles were made in a 1000 μm^2 area per sample. Identification of a synapse was based on the presence of both synaptic vesicles and post-synaptic membranous thickenings (Moriizumi et al., 1995). Synapses are readily visible using standard electron microscopic techniques.

The results of an immunohistochemical study of the characterization and ontogeny of the FMN and HMN suggested the presence or lack of synapses at specific ages in *Monodelphis* (Swanson et al., 1996). This study could not, however, positively associate the immunoreactivity with the actual presence of synapses or synaptic vesicles. Review of other studies related to the actual visualization of synapses in the brain, and how they develop, supported the idea to utilize electron microscopy to examine the FMN and HMN in order to verify if the ultrastructural anatomy corresponded to the immunoreactivity. This study has utilized specific ages of neonatal Brazilian opossum pups to see if and when synapses are present in the FMN and HMN, and to determine if there is a substantial amount of synaptogenesis occurring postnatally.

CHAPTER 3. MATERIALS AND METHODS

Animals

Neonatal Brazilian opossums were obtained from a colony maintained at Iowa State University in the Laboratory Animal Resource Center at the College of Veterinary Medicine. The original animals used to start the colony were purchased from the Southwest Foundation for Research and Education in San Antonio, TX. The adult animals were individually housed in plastic cages and maintained at 26°C on a 14:10 light:dark cycle. Water and food (Ferret Chow, Ralston Purina Co.) were provided ad libitum. Diets were supplemented every three days with fruit. In addition, mothers were supplemented with bran cereal after parturition. The animals and procedures used were in accordance with the guidelines and approval of the Iowa State University Committee on Animal Care.

In order to obtain pups, male and female opossums were paired for breeding for 14 days and then separated. The female opossums have a gestation period of 14 days (Fadem et al., 1982). Therefore, females were checked daily following separation from the males. The first day that the neonatal pups appeared was called 1 day of age postnatal (or 1PN). Because *Monodelphis* is a pouchless marsupial, acquisition of the neonatal pups was relatively quick and simple. To remove the pups, the mother was held gently by the tail and back exposing the litter on her belly. The pups were then removed by pinching at the base of the teat and carefully guiding the pups back, releasing the teat from each animal's mouth. At least three animals from two separate litters at each age were used for this study. The ages of the neonates were 5PN, 9PN, 15PN, and 25PN.

Tissue Preparation

Animals at the corresponding ages were removed from their mothers, and their heads were collected by decapitation and immediately placed into 5% glutaraldehyde fixative in 0.05M phosphate buffered saline (PBS), pH 7.2, at room temperature. The cranial skin was removed and a coronal cut was made in the area between the rostral and caudal colliculi. The caudal portions containing brainstem were placed back into fixative for 48 hours (hr) at 4°C. The specimens were then rinsed in buffer (0.05M PBS, pH 7.2), and vibratome sectioned at 100 μm on a D.S.K Microslicer (DTK-1000; Pelco) and placed into the same buffer. The sections were stained with 1.5% methylene blue for 30 seconds (s) and rinsed 4 times with buffer (Dellmann et al., 1983). This staining procedure stained the large motoneuron cell bodies darker blue than surrounding tissues and facilitated identification of the motor nuclei. The stained sections were placed on a slide and covered with buffer to avoid drying. The sections were viewed under low magnification on an Olympus stereomicroscope to identify the FMN and HMN. The sections were flipped over and examined on the opposite side in order to verify they contained FMN or HMN throughout the 100 μm thickness. This validated that the dissected tissue blocks would only contain tissue from the specific nuclei (not to include any surrounding tissue). Portions of tissue containing only FMN or HMN were dissected out using an acetone cleaned razor blade and placed into primary fixative. At least two samples of each nucleus were obtained per animal.

After all tissue blocks were collected, all vials were processed together under the same conditions. The tissue blocks were approximately $1mm^3$ or smaller, and were very difficult to see. The exchange of solutions was performed using a micropipette tip on a disposable

Pasteur pipette, and tissue blocks were visualized under the stereomicroscope. The tissue blocks were placed on a rotator at room temperature for the remainder of processing. Primary fixative was removed and the tissue blocks were rinsed in buffer three times for 15 minutes (min.) each time, and then followed by a secondary fixation step using 1% osmium tetroxide in 0.05M PBS buffer, pH 7.2, for 2 hr at room temperature. After this step, the tissue blocks were readily visible without the use of the stereomicroscope due to the brown coloration caused by the bound osmium. The secondary fixative was removed and the tissue blocks were washed in distilled water three times, 10 min. each. Then the samples were processed through a graded ethyl alcohol series of 25%, 50%, 70%, 95%, 100%, 100%, 100%, for 10 min. each step. The tissue blocks were cleared in absolute acetone and processed into pure EPON resin mixture through a graded series of acetone to resin mixtures, 3:1, 1:1, 1:3, and finally into pure EPON resin mixture. Each infiltration step was for 24 hr with an additional 24 hr in pure EPON resin mixture prior to casting. All tissue blocks were cast individually into tapered beam capsules and given sequential identification numbers. A separate log was made to include the identification number, the litter number, age, and appropriate motor nucleus for each tissue block.

Two tissue blocks from each animal (one facial and one hypoglossal) at each age were sectioned using a Reichert ultramicrotome. Each block was sectioned at two different levels. The first level was taken 40-50 μm into the tissue block. Thick sections (1 μm) were placed on slides, stained with methylene blue, coverslipped, and viewed on a Leitz light microscope. The thick sections were used to verify that the tissue block was from the correct area and that electron microscopic evaluation of subsequent sections corresponded to the area of interest.

Thin sections (60-80 nm) were made using a diamond knife on a Reichert ultramicrotome. Sections were collected on Formvar coated 50-mesh copper grids and post stained with 3% methanolic uranyl acetate followed by Sato's lead stain. The second series of sections were taken 15 μm deeper into the tissue block. This same procedure for thick and thin sectioning was standard for the second level. Two areas of the individual tissue blocks were evaluated for this study.

Electron Microscopy

Sections were viewed and photographed using an Hitachi 500 transmission electron microscope. Areas were photographed using these guidelines: visualization of motoneurons with surrounding neuropil to verify location; only one resin section was photographed per grid (to avoid overlap by photographing same area on another sequential resin section); area photographed was free of wrinkles, folds, precipitate, or grid bars; and negatives were taken following a path around motoneurons without overlapping. Each section area was photographed at 10,000 magnification. Ten pictures were taken from each section area. Each negative was numbered and logged corresponding to the litter number, age of animal, and location (FMN or HMN).

Data Collection and Analysis

Synapse counts and analysis

Photographic prints (8X10") were made of each negative. The prints were mixed and then evaluated randomly without knowledge of age or location. Each synapse was identified and counted using these guidelines: there should be a membrane thickening, and the presence of three or more synaptic vesicles nearby (as previously adopted by Moore and Bernstein,

1989). Every synapse that was counted was marked with a red dot. All the dots were counted on each print and that count was logged with the corresponding negative number. After all prints were evaluated and counts were logged, the negative numbers were reallocated with the litter number, age, and location. The average number of synapses per 1200 μm^2 area was calculated for each age. An analysis of the variance statistical test was used to test for a main effect with age, location, and the interaction between both.

Cell size analysis

Slides containing thick 1 μm sections stained with methylene blue were utilized. Two ages, 5PN and 25PN, were examined. Images were collected on an Axiophot Zeiss light microscope and Sony video system using the 40X objective with the 2.5 optivar for a final magnification of 100X. The images taken were analyzed using a Vaytek image analysis system running on a Silicon Graphics Inc. workstation. Two cell bodies were outlined per slide, two slides per nucleus, at both ages, and area measurements were collected. Interpolation of the area measurements gave representative diameter estimates for the cells.

Vesicle analysis

A qualitative analysis of the number of synaptic vesicles counted was conducted for both nuclei at 5PN and 25PN. The total area evaluated was 240 μm^2. The data was collected from the same prints used in the synaptic profiles evaluation. Two prints from each level for each age were chosen randomly (from the forty available) for both nuclei. The vesicles were counted and the numbers were logged before corresponding identification numbers were reallocated with them. Each photo represented an area of 60 μm^2 for a 240 μm^2 total area evaluated.

CHAPTER 4. RESULTS

General Anatomy

The Brazilian opossum was an easy animal to work with. Obtaining pups was relatively simple at all the ages sampled. When the animals were removed at 5PN and 9PN, a small amount of blood was observed in the corners of the mouth of the neonates. This could be in conjunction with tearing of the attachment of the pups mouth by the epitrichium.

Once the heads were fixed, they were washed in buffer to reduce inhalation of the fixative during collection of vibrotome sections. The brain tissues appeared yellow-tan and were very firm. The 5PN brains were not dissected out of the skulls. The entire heads were sectioned due to their small size. The vibrotome sections were firm and easy to handle with a camel hair brush. After the sections were stained and placed on a slide, the sections containing FMN or HMN were removed. Figure 1, A-D shows 100 μm sections stained with methylene blue containing FMN and HMN at 5PN and 25PN. Although the sections were thick, it was possible to identify the prominent nuclei of the motor neuron cell bodies while focusing through a section. Dissection of the motor nuclei was difficult at 5PN since they were only 0.5 mm to 1.0 mm in size. The tissue blocks were very small and the use of a dissecting microscope was necessary throughout the initial processing.

The resin-embedded tissue appeared well fixed (Figure 2, A-H). The identification of the motoneurons was very easy due to their prominent nuclei and nucleoli (Figure 2, A-H). Examination at the electron microscopic level revealed some minor fixation damage. Membranes and organelles appeared intact although occasionally some mitochondria were

Figure 1. Light micrographs representing 100 μm thick vibratome cut sections of the FMN and HMN at 5PN and 25PN. Nuclei are surrounded by a dashed line indicating perimeter from which nuclear material was dissected, bars = 500 μm. A: 5PN FMN, cartilage (c) is evident directly beneath brainstem containing the FMN. B: 25PN FMN. C: 5PN HMN, fourth ventricle (4v) is evident. D: 25PN HMN.

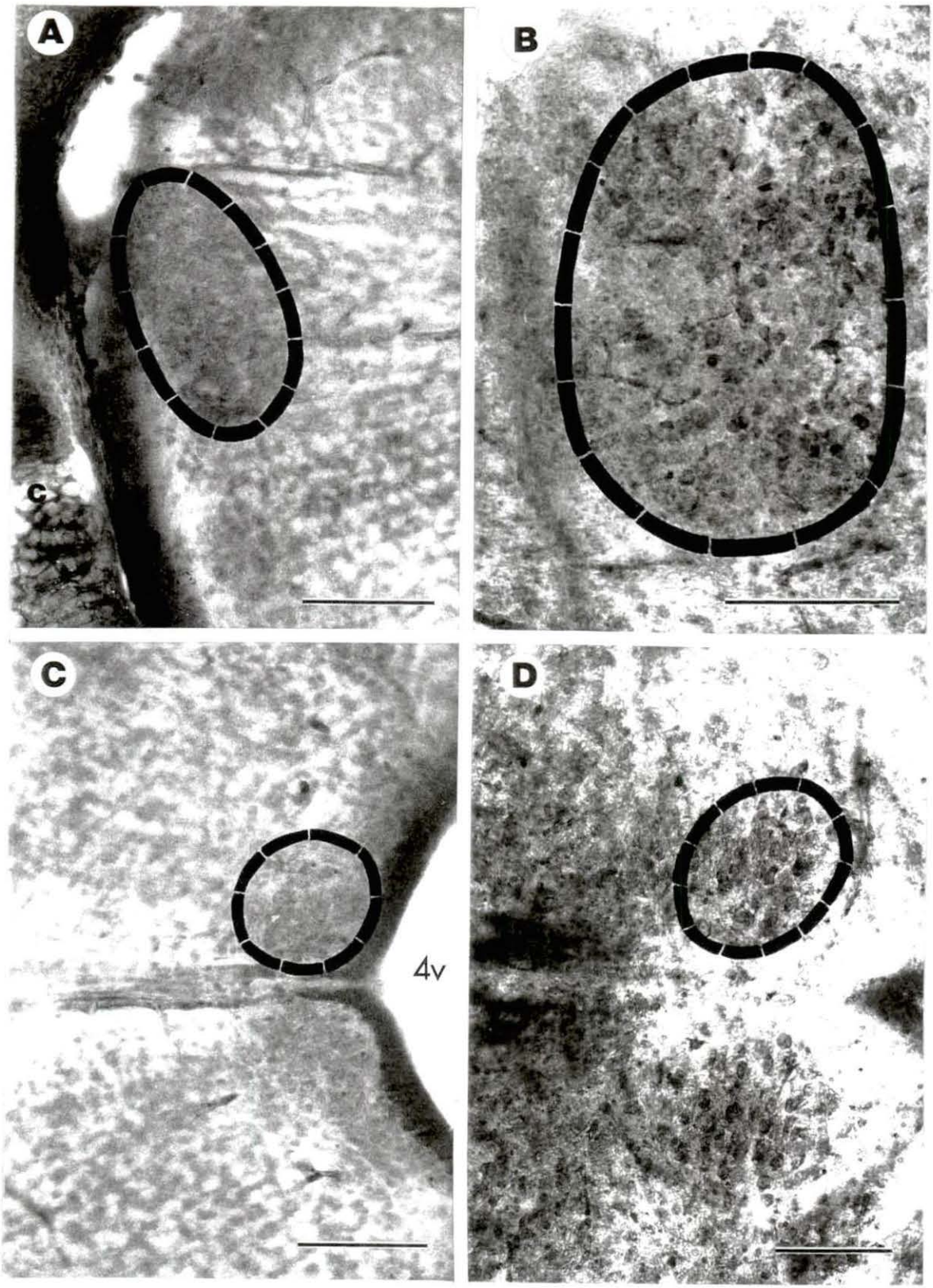
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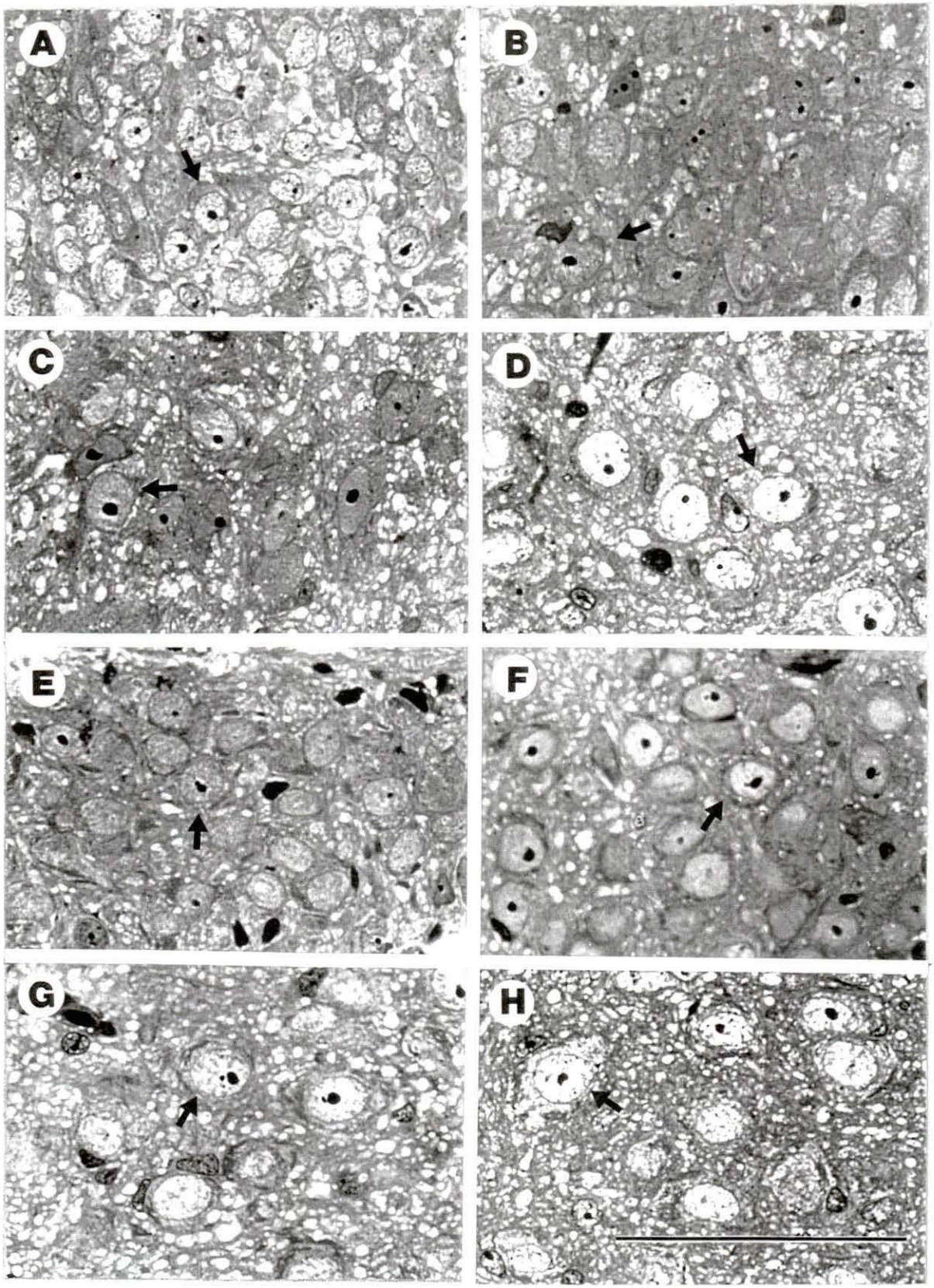
Figure 2. Light micrographs of FMN and HMN at ages 5, 9, 15, and 25PN, bars = 20 μm, arrows indicate large motoneuron cell bodies (soma). A: 5PN FMN. B: 9PN FMN. C: 15PN FMN. D: 25PN FMN. E: 5PN HMN. F: 9PN HMN. G: 15PN HMN. H: 25PN HMN.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disrupted. The ultrastructural appearance of both motor nuclei was unusual due to the abundant cross sections through dendrites. The proximal dendrites were very large and contained very small amounts of cellular material creating a holey appearance in the tissue. The cell bodies at 5PN appeared closely packed as compared to 25PN. There was less neuropil in the younger ages than in the older ages in both the FMN and HMN. The synapses were easily identifiable due to their affinity for the heavy-metal counterstains used. Figures 3 through 10 are examples of each age from both the FMN and HMN. Figure 11 shows high magnification micrographs of synapses from both nuclei at 5PN and 25PN. The post-synaptic density, the synaptic cleft, and the presence of synaptic vesicles were evident. The types of synaptic vesicles observed varied including spherical, flattened, pleomorphic , and large dense-core vesicles.

Synapse Evaluation

Synapses were counted and the numbers were logged. The data containing the synaptic profile counts taken from the electron micrographs is presented in Table 1. Each number logged is an average of the four animals; each animal had a sum of 20 prints counted, 10 from each level or grid. Examples of one of the 10 electron micrographs taken from each level in both the FMN and HMN at each age are presented in Figures 3 to 10. Each 8x10" print covered an area of about 60 μm^2. The total area evaluated for each nucleus in each animal is 1200 μm^2. It is evident that postnatal synaptogenesis is occurring in both the FMN and HMN (Table 1; Figure 12). In the FMN, the average number of synaptic profiles per 1200 μm^2 area increased 2.7 times from 5PN to 25PN. In the HMN, the number increased 1.4 times from 5PN to 25PN.

Figure 3. Electron micrograph of 5PN FMN, arrows indicate countable synapses, bar = 2 μm.

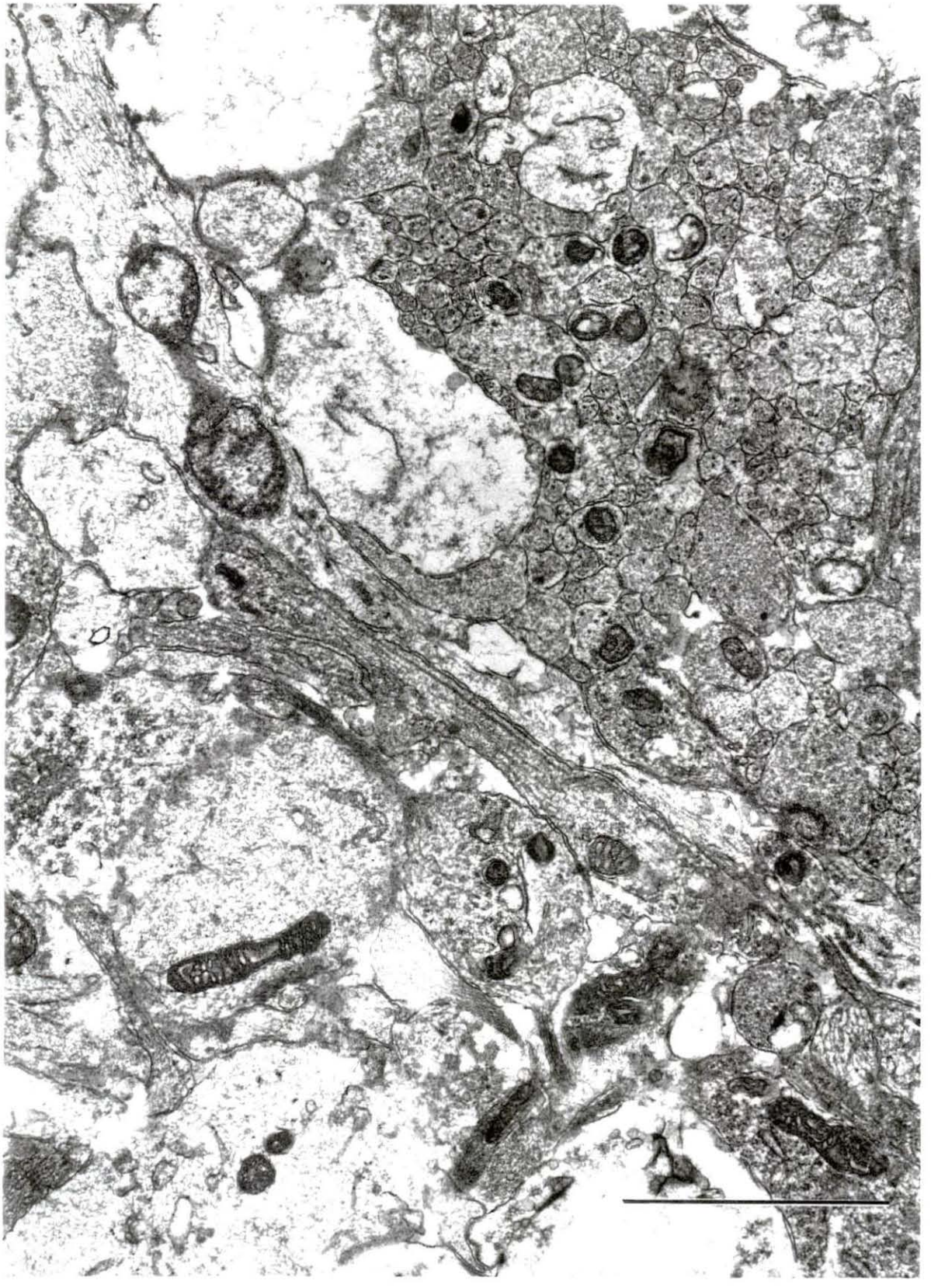

Figure 4. Electron micrograph of 9PN FMN, arrows indicate countable synapses, bar = 2 μm.

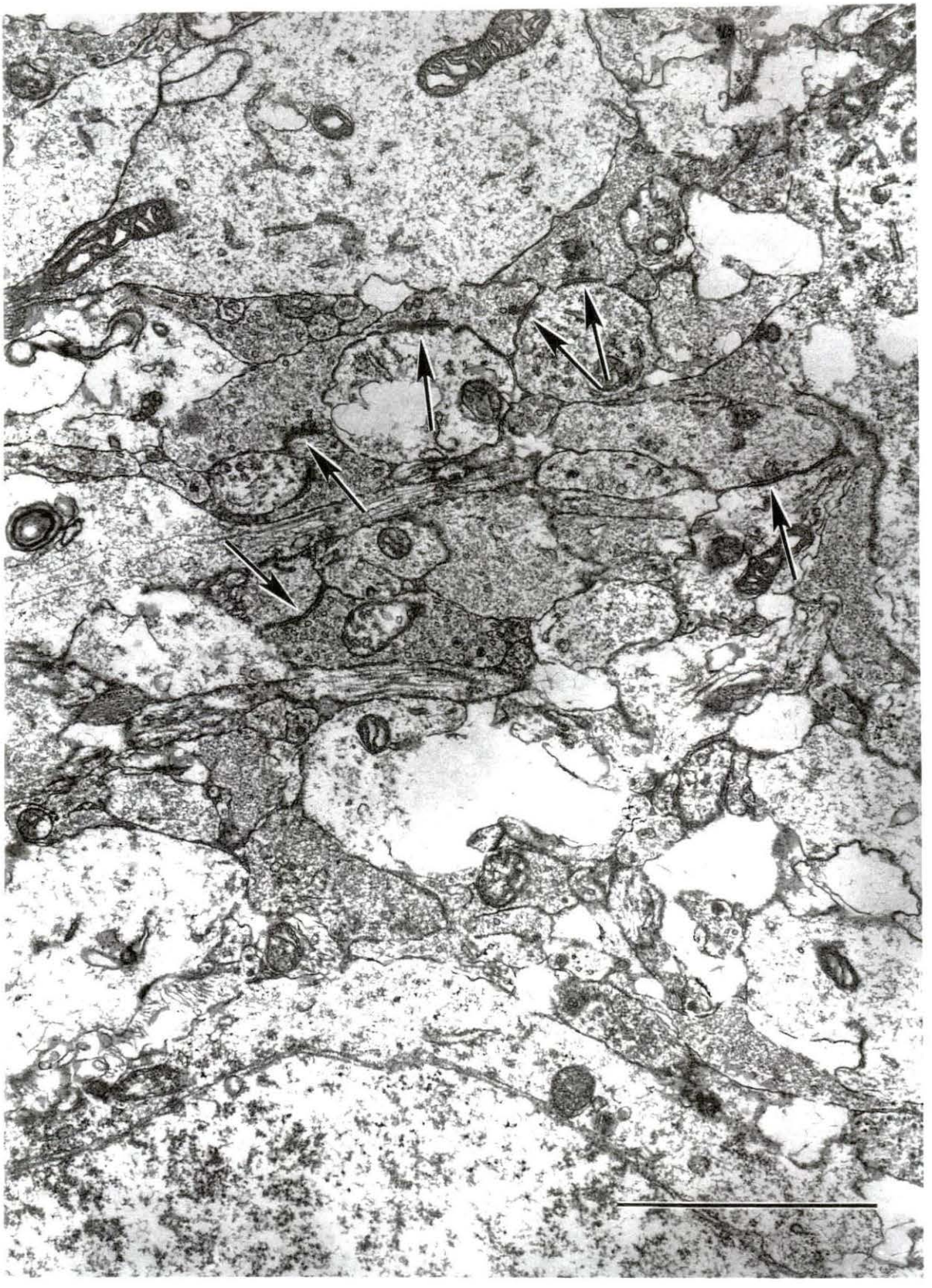

Figure 5. Electron micrograph of 15PN FMN, arrows indicate countable synapses, bar = 2 μm.

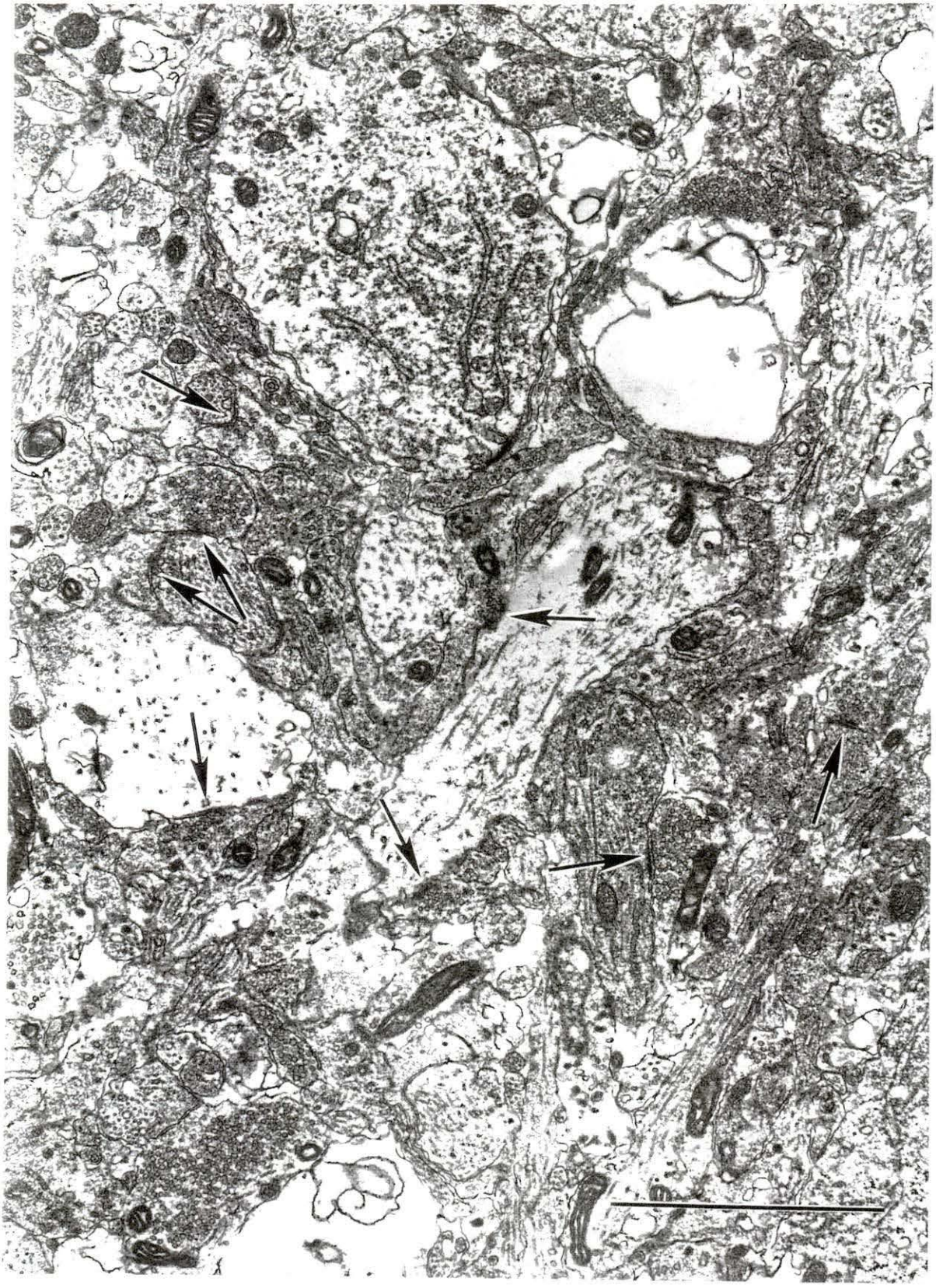

Figure 6. Electron micrograph of 25PN FMN, arrows indicate countable synapses, bar = 2 μm.

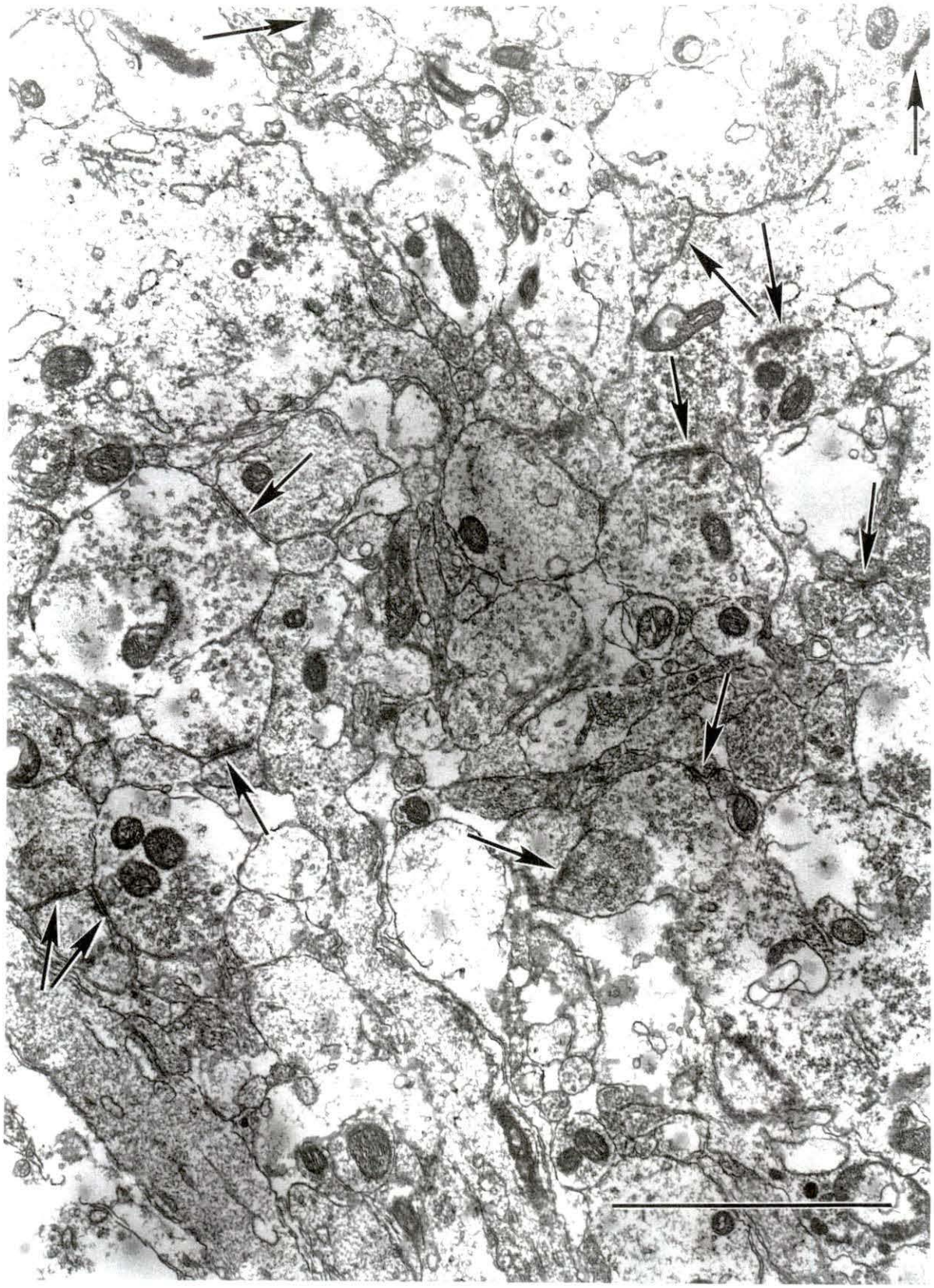

Figure 7. Electron micrograph of 5PN HMN, arrows indicate countable synapses, bar = 2 μm.

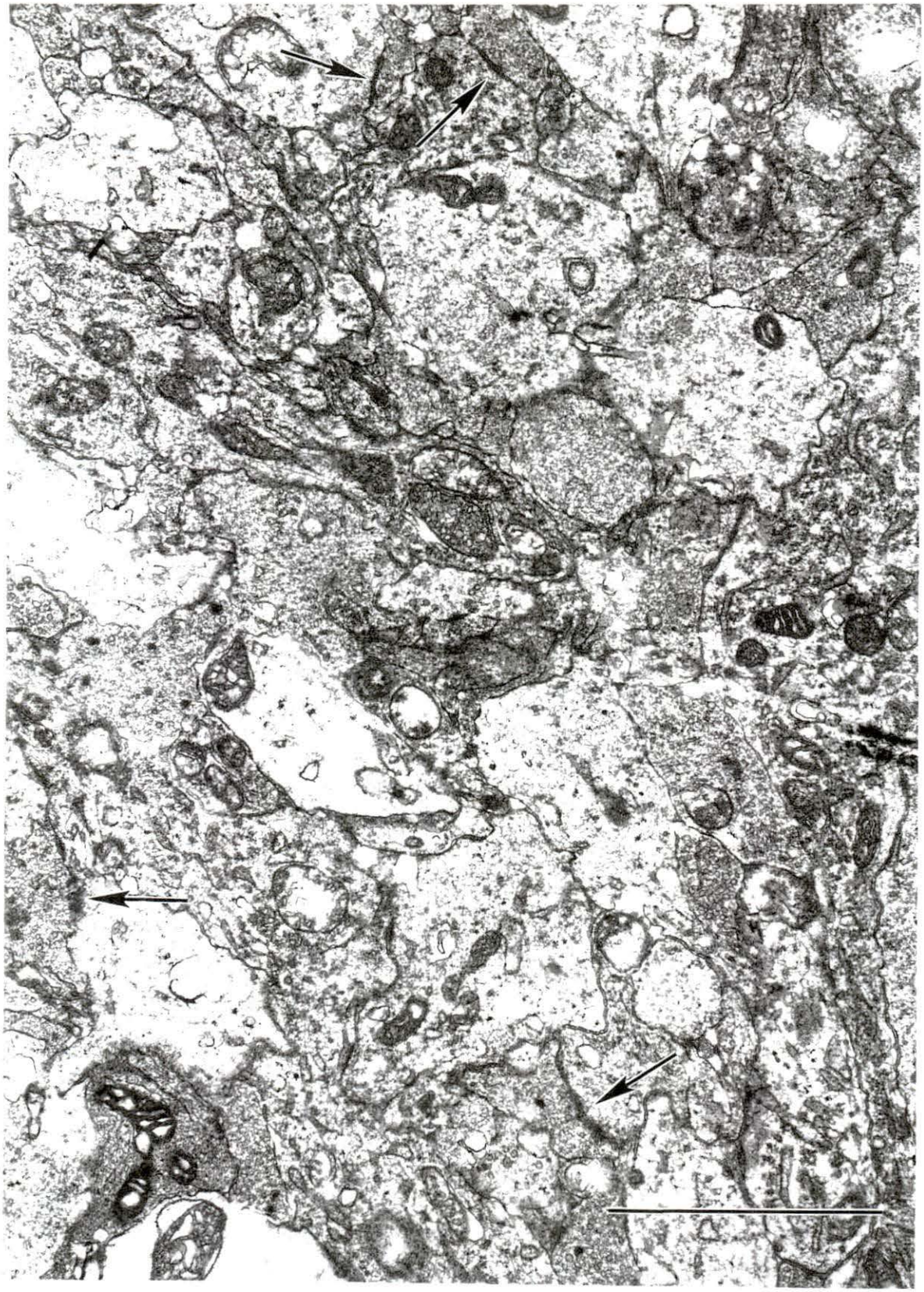

Figure 8. Electron micrograph of 9PN HMN, arrows indicate countable synapses, bar = 2 μm.

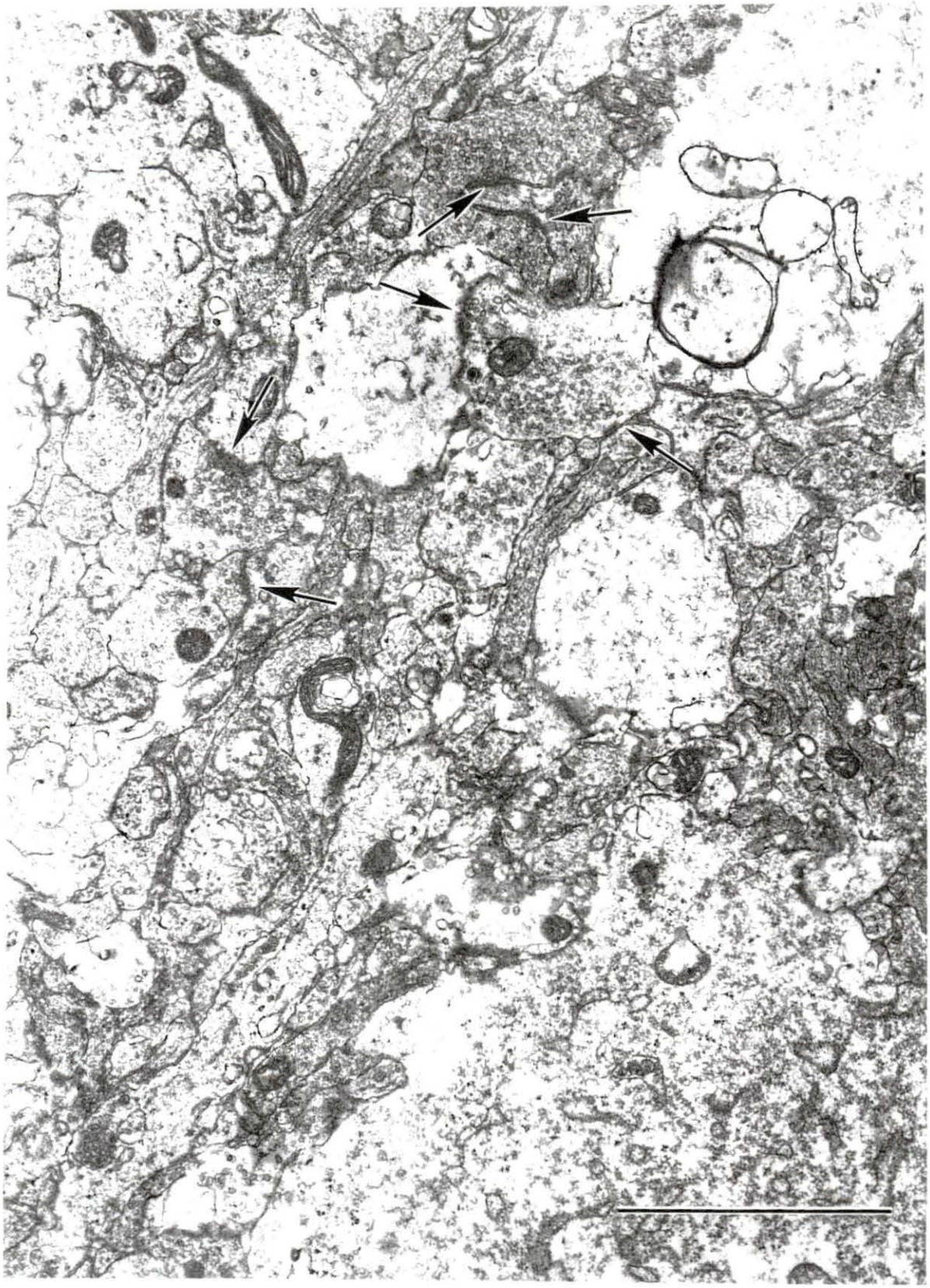

Figure 9. Electron micrograph of 15PN HMN, arrows indicate countable synapses, bar = 2 μm.

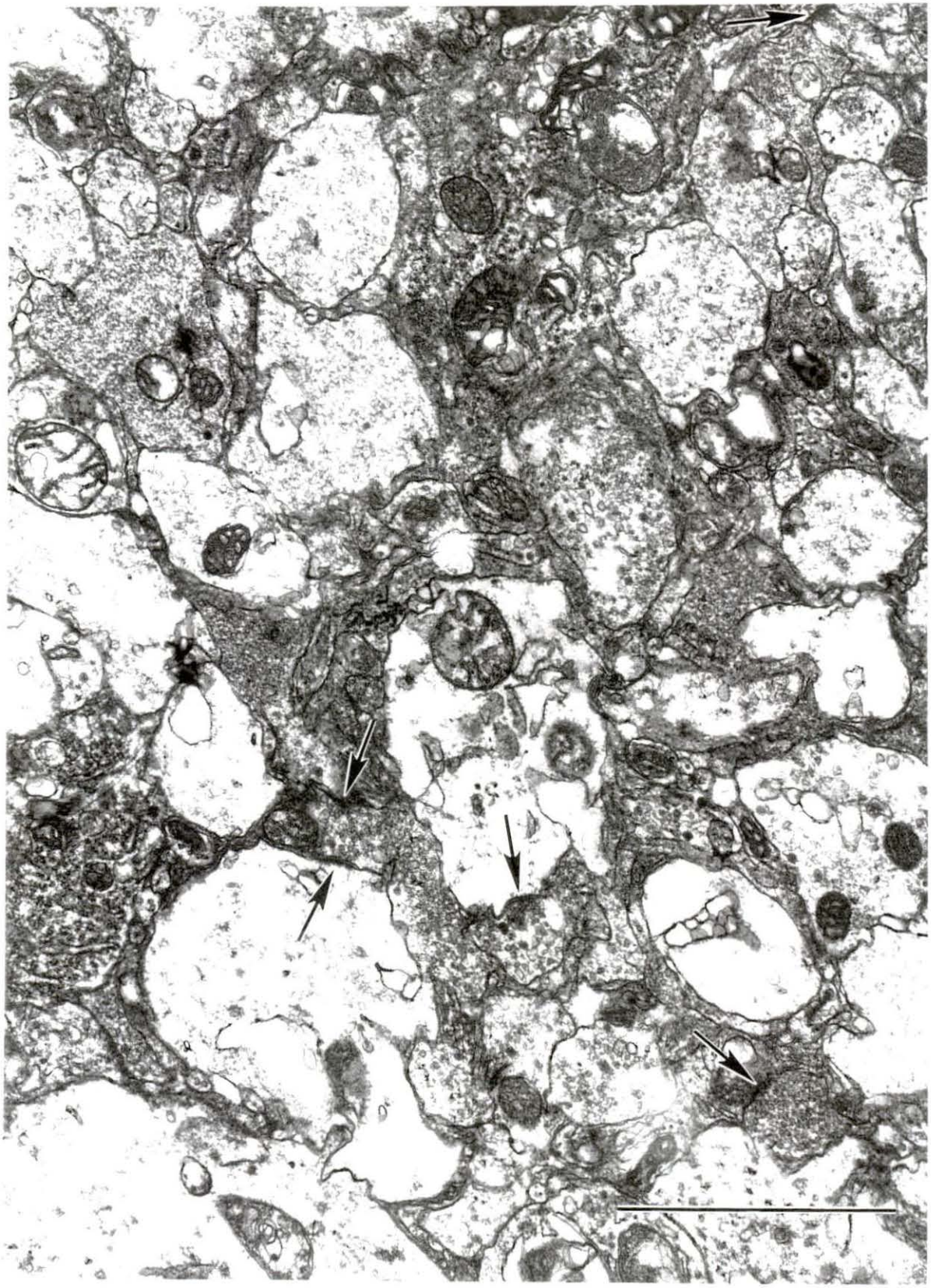

Figure 10. Electron micrograph of 25PN HMN, arrows indicate countable synapses, bar = 2 μm.

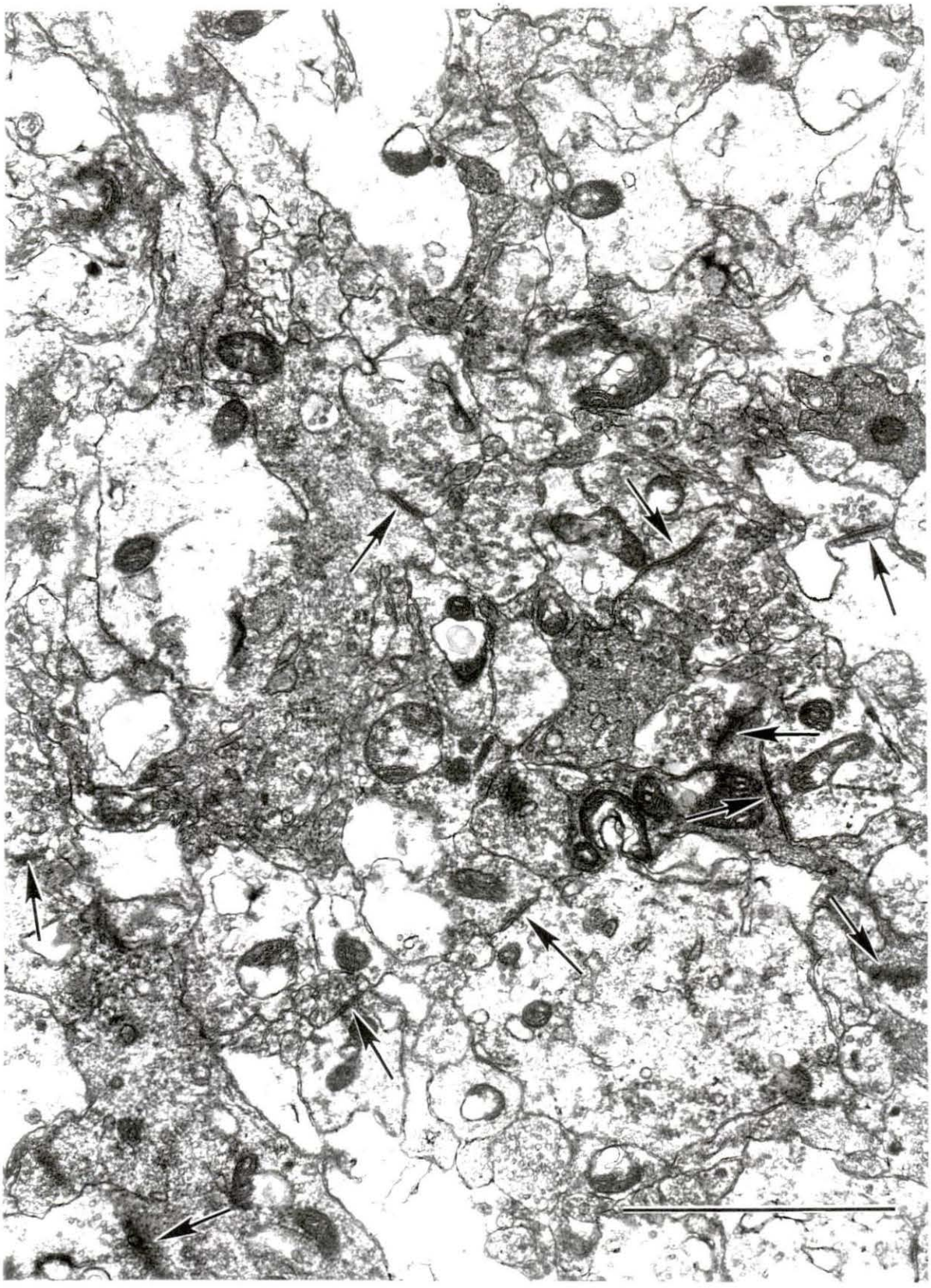

Figure 11. Electron micrographs of synapses in the FMN and HMN at 5PN and 25PN, arrows indicate synapses with associated synaptic vesicles, post-synaptic densities, and synaptic clefts, bars = 500 nm.

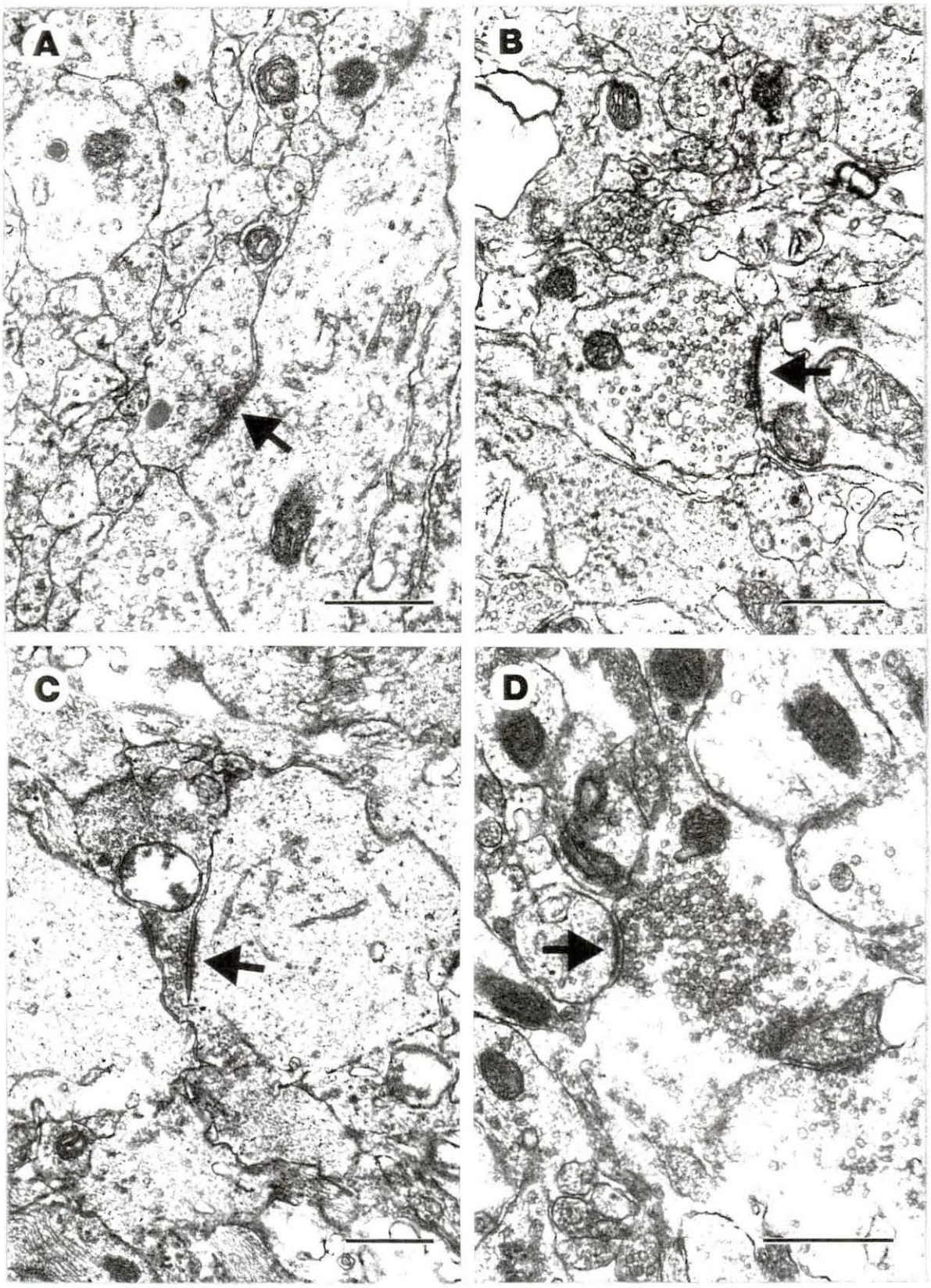
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Table 1. Summation of the counts of synaptic profiles.

Age	Average number of synapses counted per 1200 μm^2 area in the FMN with standard error	Average number of synapses counted per 1200 μm^2 area in the HMN with standard error
5PN	**61.75 ± 10.20**	**81.66 ± 4.90**
9PN	**114.66 ± 21.20**	**108.75 ± 41.23**
15PN	**169.00 ± 26.54**	**114.50 ± 18.71**
25PN	**216.00 ± 7.63**	**205.00 ± 20.50**

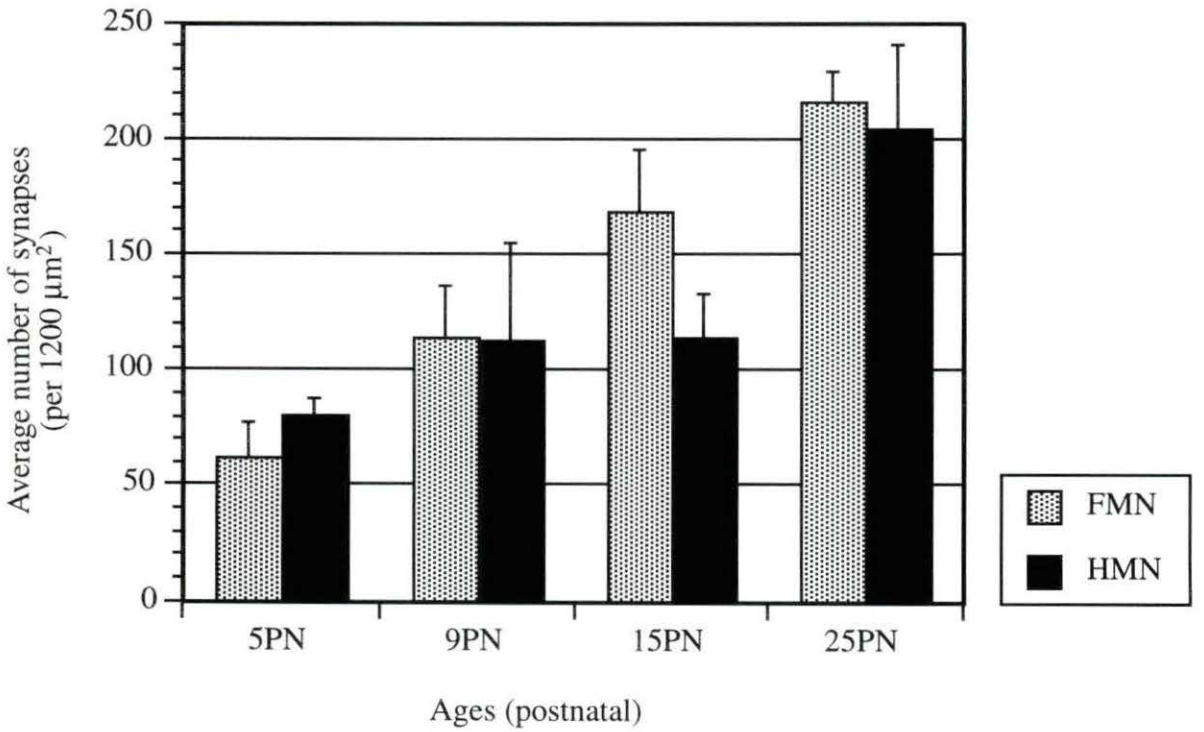

Figure 12. Histogram of average number of synaptic profiles counted in a 1200 μm^2 area at 5PN, 9PN, 15PN, and 25PN for FMN and HMN. Light bars represent FMN and dark bars represent HMN. Number of synaptic profiles increases with age in each nucleus.

Statistical analysis (SAS-ANOVA) showed an effect was evident with increasing age in the average number of synaptic profiles counted. There was no effect evident with location (between the FMN and HMN) or with an interaction between the age and location indicating that there was a significant amount of development occurring in both motor nuclei with age, but not differentially between the two.

Cell Size Evaluation

The cell size analysis was performed on slides containing 1 μm thick resin sections stained with methylene blue. The image analysis system digitized the images captured from the light microscope with video. The images were filtered to increase contrast so that the cell bodies were identifiable and could be traced. The areas were interpolated into a diameter estimate for analysis.

There was an apparent increase in the size of the motoneuron cell bodies with age (Table 2; Figure 13). The data represents the interpolated area measurements taken from cell soma in the FMN and HMN. The numbers represent the estimated diameter based on the area measurements taken using Visilog image analysis system software (the area totals are in square microns, μm^2). The bold numbers are an average of the measurements for each age. There is a significant difference in the average diameter of the cells between 5PN and 25PN in the FMN and in the HMN. However, there is no indication that there is any difference between the FMN and HMN at 5PN or at 25PN.

Table 2. Cell size analysis data.

Age & Area	Average Cell Diameter (in microns) with standard error
5PN FMN	**13.03 ± 0.3326**
25PN FMN	**18.93 ± 0.5446**
5PN HMN	**14.18 ± 0.3632**
25PN HMN	**20.78 ± 0.2015**

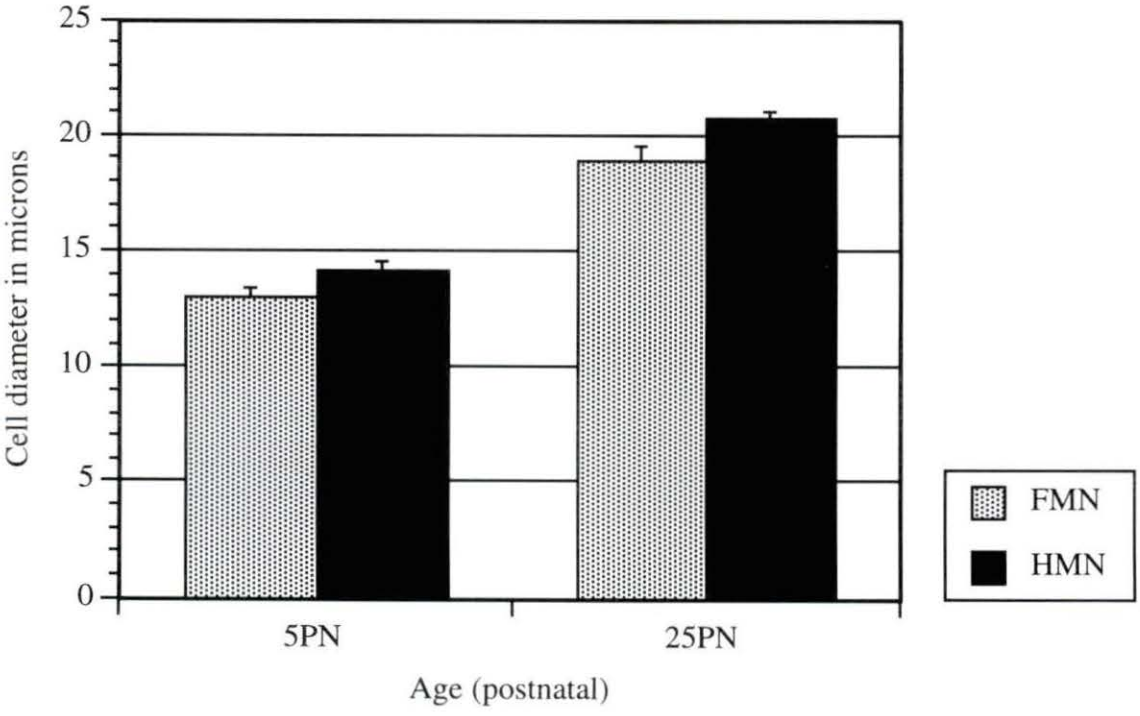

Figure 13. Histogram of the average cell diameter measured in HMN and FMN. An apparent increase in cell diameter is evident with age but not between FMN and HMN.

Synaptic Vesicle Evaluation

All types of synaptic vesicles were counted for this analysis including spherical, flattened, pleomorphic, and large dense-core vesicles. The vesicles associated with a synapse were counted along with vesicles not associated with a synapse. It was important to get an estimate of the entire amount of vesicles present in a given sample area in order to compare the vesicle number to the amount of immunoreactivity of the synaptic vesicle associated proteins in the immunohistochemical study (Swanson et al., 1996).

The number of synaptic vesicles were counted in the FMN and HMN at 5PN and 25PN in a specific area of 240 μm^2. There was an apparent increase in the number of synaptic vesicles occurring with increasing age (Table 3; Figure 14). At the younger ages the synapses appeared to have prominent membrane densities but fewer associated vesicles. There did not seem to be a difference between the two nuclei at similar ages. There was no apparent difference in the type of vesicles observed between the two nuclei. Figure 11 shows examples of synapses and associated vesicles at 5PN and 25PN in both the FMN and HMN.

Table 3. Synaptic vesicle counts.

LOCATION	5PN	25PN
HMN	**801**	**3270**
FMN	**1161**	**3347**

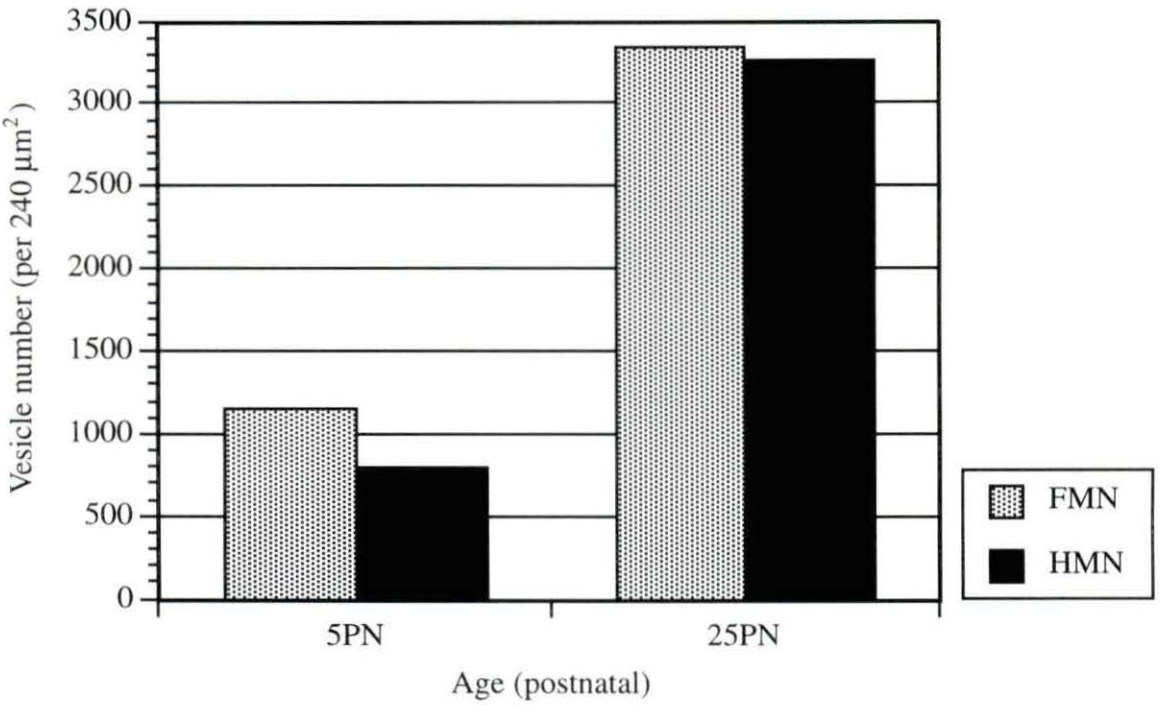

Figure 14. Histogram of data presented in Table 4 representing number of synaptic vesicles counted in a 240 μm^2 area in both HMN and FMN at 5PN and 25PN. There is a notable increase in number of synaptic vesicles with age but not a significant difference between both motor nuclei.

CHAPTER 5. DISCUSSION

This study has examined the FMN and HMN at the electron microscopic level during early stages of development in *Monodelphis domestica*. After reviewing previous studies involving these specific motor nuclei, the question was raised as to whether varying levels of immunoreactivity for synapse and synaptic vesicle associated proteins seen in the FMN and HMN of *Monodelphis* (Swanson et al., 1996) corresponded to the presence of synapses at the ultrastructural level. The results of this ultrastructural examination were consistent with the theory that there is a marked amount of development occurring postnatally in the FMN of *Monodelphis*. The results of this study also support the concept that the HMN is undergoing a substantial amount of development as well.

Three different aspects of development were evaluated in the FMN and HMN of *Monodelphis*. The first aspect involved determining the average number of synapses in a specified area (1200 μm^2) with the electron microscope in both motor nuclei at all ages sampled: 5PN, 9PN, 15PN, and 25PN. This study began with age 5PN, based on a previous study by Swanson et al. (1996), which indicated that motoneurons of the FMN did not appear in the area of the FMN until 5PN. The second aspect involved examining the motoneuron cell bodies within the FMN and HMN. This involved obtaining an estimation of the soma size at 5PN and 25PN to assess whether there was any apparent growth occurring, or if there was any observable difference between the FMN and HMN. The cell size study utilized ages 5PN and 25PN from both motor nuclei. The final aspect involved examining a same size area within both the FMN and HMN and counting the number of synaptic vesicles (regardless of type or location) present to determine if there was any increase with age or if there was any

over all difference between the motor nuclei. The synaptic vesicle study utilized ages 5PN and 25PN from both motor nuclei.

Synaptogenesis

The synaptogenesis study examined the average number of identifiable synaptic profiles in a consistent sample area (1200 μm^2). This evaluation revealed a marked increase in the average number of synapses between the ages of 5PN and 25PN in both the FMN and HMN. It is interesting to note the substantial amount of postnatal development occurring in the FMN and HMN. These animals are able to suckle at birth and it is unusual that neuronal systems involved in regulating motor function of muscles associated with suckling behavior are still developing. There was a significant difference in the average number of synapses present from 5PN to 25PN ($p>0.001$ in the FMN and $p>0.004$ in the HMN). The increase in the average number of synapses seen with age could be due to innervation of both motor nuclei by afferents coming from various midbrain, pons, and medullary centers as these specific regulatory centers mature and grow. Included in this concept is the possibility that the appearance of synapses corresponds to branching of afferent projections already present in the nuclei, but expanding their communication network throughout. The muscles of the tongue and peripheral muscles of the face may undergo a substantial amount of development as well, either increasing in fascicle number or increasing in size which would indirectly cause the need for increased innervation of the musculature. An increase in musculature would directly require more regulation and thus increase the appearance of synapses in the corresponding motor nuclei.

Although delayed synaptogenesis observed in the FMN was expected, the appearance of synapses at 5PN was rather unexpected based on previous studies by Swanson et al. (1996). None of the synapse associated immunohistochemical protein markers exhibited positive reactivity at 5PN in the FMN (Swanson et al., 1996). Actually, the FMN did not have any positive immunoreactivity until 15PN (Swanson et al., 1996) suggesting a lack of or a reduced number of synapses appearing until then. However, in the HMN there was positive immunoreactivity present at 5PN suggesting a greater amount of synapses should be present at this age compared to the FMN. Therefore, the substantial increase in the average number of synapses evident with age in the HMN at the electron microscopic level was unexpected. It is possible the proteins that were utilized in the study by Swanson et al. (1996) were only a subset of proteins associated with synapses and synaptic vesicles. Relatively the same amount of synapses were evident at 5PN in both motor nuclei suggesting that these specific protein markers may be present only in functional synapses as opposed to being anatomically associated.

When the average number of synapses were compared at 15PN, the average number of synapses in the FMN exceeded that of the HMN. In fact, the HMN appeared to have little growth from 9PN to 15PN. There are a few possible explanations for this unusual observation. This lack of synaptogenesis in the HMN possibly corresponds to differential afferent innervation. The FMN could be receiving more constant innervation whereas the HMN may reach a maximum level at this age until further growth occurs in the afferent centers or a greater amount of regulation of the musculature is needed. Although unlikely, the reduced synaptogenesis at 15PN in the HMN may correspond to the HMN having fewer cell

bodies and, therefore, fewer processes with which afferents could form synapses. Since the FMN has more cell bodies present, afferent innervation may not slow down. By 25PN the HMN has reached almost the same average number of synapses as the FMN. This is a rapid increase in synaptogenesis during the time from 15PN to 25PN in the HMN. This again could directly correspond to an increase in afferent innervation due to growth of the afferent centers or growth of the tongue musculature. It is interesting to note a marked amount of postnatal development in these motor nuclei directly related to the suckling behavior of neonates.

Cell Size and Synaptic Vesicles

The cell size analysis evaluated the size (diameter estimate) of the motoneuron cell bodies in the FMN and HMN at 5PN and 25PN. The results of this analysis indicated an increase in the estimated diameter of the motor neuron cell bodies in both the FMN and HMN from 5PN to 25PN. There was, however, no difference between the FMN and HMN at either age. This apparent increase in cell body size in both motor nuclei supports the idea that the data collected for the synaptogenesis study is unbiased. If there was a difference in motoneuron growth rate between the FMN and HMN, the data from the synaptogenesis study could have been biased based on one nucleus having more area for synapses to occur. Because there is no motoneuron size difference evident between the FMN and HMN, this study supports the idea that a great amount of postnatal synaptogenesis is ongoing in both motor nuclei.

The synaptic vesicle study examined the number of synaptic vesicles in a specified area (240 μm^2) at 5PN and 25PN in both motor nuclei. An increase in the number of synaptic vesicles was apparent from 5PN to 25PN in both the FMN and HMN. There was no

difference evident between the FMN and HMN in the number of vesicles at either age. In an immunohistochemical study by Swanson et al. (1996), positive reactivity for specific synaptic vesicle associated proteins occurred in the HMN at 1PN and in the FMN at 15PN. The results of this study at the ultrastructural level do not compare with the results of the study by Swanson et al. (1996). It is possible that the antigenicity of the protein markers may have been insufficient enough to detect the fewer numbers of vesicles present at the younger ages as observed ultrastructurally. It is also possible that previous types of protein markers utilized for immunohistochemical studies were only a subset of proteins present in synaptic vesicles. The protein markers may have been functionally related as opposed to anatomically related. Therefore, only synaptic vesicles that were being utilized would cause a positive reaction. This explains the lack of difference in synaptic vesicle number between the FMN and HMN at 5PN. The increasing number of synaptic vesicles from 5PN to 25PN suggests that both the FMN and HMN are undergoing a substantial amount of postnatal development.

Synaptogenesis and the Suckling Response

It is apparent that the CNS of neonatal *Monodelphis domestica* is significantly underdeveloped as compared to placental mammals. The unique ability of this marsupial animal to survive in such an immature state has led us to try to understand how different systems develop that are directly involved in the survival of these animals. The motor systems involved in the suckling behavior of *Monodelphis* would be expected to be very well developed since utilization of this system is required within the first 24 hr after birth. When considering the data collected in this study, two explanations for this phenomenon are apparent. The first theory suggests that the actual number of synapses is not directly related

to the function of the motor nuclei. There could be other regulators assisting in the function of the systems involved in the suckling response while the FMN and HMN develop. This possibility is substantiated by the fact that facial motoneurons express cholecystokinin (CCK) binding sites (Kuehl-Kovarik et al., 1993) while no CCK-immunoreactive fibers are detected (Fox et al., 1991) suggesting parasynaptic flow. The second theory suggests that the facial muscles are not needed in order for the neonates to receive nourishment from the mothers. It could be that the oral tube, formed after the pups locate and attach to a teat, is simply a vessel for collection of the milk expulsion from the mother brought on by the pump-sucking mechanism involving only the tongue. The facial musculature may not be needed until after the thin epitrichium that attaches to the corners of the mouth has atrophied. There is the potential for various explanations as to how these motor systems function under such immature development. In any case, it is apparent that synaptogenesis is occurring postnatally in both the FMN and HMN.

CHAPTER 6. CONCLUSIONS

Electron microscopic evaluation revealed a marked increase in synapses between the ages of 5PN and 25PN in both the FMN and HMN of *Monodelphis domestica*. An obvious difference in the number of synaptic profiles does not exist between the FMN and HMN. Thus, there seems to be a discrepancy between previous studies by Swanson et al. (1996) examining immunoreactivity in the FMN and HMN and the results found at the electron microscopic level. In this study, an increase in the number of synaptic vesicles was observed ultrastructurally with age but not between the FMN and HMN. Examination of synaptic vesicles indicated an increased number with increasing age. Similarly, an increase in the cell body size was observed with age in both nuclei, but no difference was observed between them. It is evident that synaptogenesis is occurring in both motor nuclei in postnatal animals. It is significant that synaptogenesis is ongoing in oromotor nuclei potentially utilized by the suckling neonate.

Some important questions were raised during this study that propose future studies which may explain why such discrepancies occurred between this ultrastructural study and the immunohistochemical study by Swanson et al. (1996). Would it be possible to elucidate the timing of innervation patterns in the nuclei corresponding to afferents from known centers in the brainstem and midbrain? This could potentially explain which afferents are forming synapses in the FMN and HMN, when they appear, and any differences between the nuclei. Would it be possible to visualize functional synapses at the level of the neuromuscular junction ultrastructurally, thus indicating if and when efferent innervation occurs in the facial muscles, and in the muscles of the tongue? This could answer the question of whether or not these

muscles are actually needed for survival immediately after birth. There are still many features of the developing oromotor system that remain unexplained. One aspect is very clear; synaptogenesis is ongoing in the FMN and HMN of the neonatal Brazilian opossum.

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