ABNORMALITIES OF THE DORSAL MOTOR NUCLEUS OF THE VAGUS IN SUDDEN INFANT DEATH SYNDROME

by

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Abstract

Recent hypotheses concerning the pathogenesis of sudden infant death syndrome (SIDS) have focused on developmental abnormalities of the central nervous system, which are believed to produce cardiorespiratory instability in susceptible infants. The present study was designed to investigate developmental abnormalities in the dorsal motor nucleus of the vagus (DMV) in SIDS.

Using serial histological sections of the brainstem from SIDS cases and age-matched control cases without neurological disease, morphometric analyzes were performed to compare postnatal changes in 1) the total volume of the DMV, 2) the numerical density of neurons (N_V, cells per mm^3), 3) the total number of neurons, and 4) mean neuronal profile area. In the SIDS cases there was a significant increase in the total volume of the DMV (33%) , when compared to controls. In SIDS there was a significant decrease in the N_V of neurons (33%), although the total number of neurons did not differ significantly from controls. Mean neuronal profile areas were significantly greater in SIDS for both motor (31&) and non-motor (30%) neurons, when compared to controls. These changes are consistent with an overgrowth of the DMV during early postnatal development. Given the role of the DMV in the autonomic control of breathing and heart rate, this subtle developmental disorder likely contributes to the cardiorespiratory instability characteristic of susceptible infants.

Given the possibility that increased expression of insulin-like growth factor I during early postnatal development might contribute to this overgrowth, morphometric analyses were performed on the DMV and hypoglossal nucleus (HN) in transgenic mice, which overexpress IGF-I postnatally, and in normal littermate controls on postnatal day 35. Morphometric variables included 1) the total volumes of the DMV and HN, 2) the N_V of neurons, 3) the total number of neurons, and 4) the mean neuronal profile areas. In transgenic mice there was a significant increase in the volumes of both the DMV (84%) and the HN (30%). The N_V of neurons was significantly reduced in both nuclei in transgenic mice. In the DMV, however, there was a significant increase in the total number of neurons (56%). In the HN, the total

number of neurons did not differ significantly between transgenic mice and controls. Mean neuronal profile areas were significantly increased in transgenic mice in both the DMV (35%) and the HN (22%). Available evidence suggests that the increased neuron number in the DMV results from an antiapoptotic effect of IGF-I.

In a third experiment, morphometric and stereological analyses were performed in the DMV of rats during normal postnatal development to determine the time course of the progressive and regressive phases of synaptogenesis. The initial phase of synapse proliferation occurred from birth to postnatal day 30, while the regressive phase of synapse elimination occurred after day 30. Recent hypotheses suggest that SIDS results from a failure to eliminate normally extraneous synapses from the brainstem. By knowing the age at which peak synaptic densities occur, one could introduce exogenous growth factors to prevent normal elimination of synapses. Preventing synapse elimination from the brainstem of the rat might be expected to produce an animal model of SIDS.

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Chapter 1.

Introduction.

1.1. An overview of sudden infant death syndrome

1.1.1. Historical look at SIDS

Sudden infant death syndrome (SIDS) is defined as "the sudden death of an infant between the ages of one month and one year of age, which remains unexplained after a thorough case investigation including a complete autopsy, examination of the death scene, and review of the clinical history", (Willinger et al., 1991). SIDS is the most common cause of infant death with an incidence of 1-2 cases per 1000 live births (Schellscheidt et al., 1997; Becker, 1990).

Although the concept and definition of SIDS have only been formally recognized since 1969 and the diagnosis of SIDS as a cause of death has only been official since 1979 (Culbertson et al., 1988), sudden infant death syndrome has been occurring for many centuries. Documentation of sudden death in infants dates back to biblical times. Written in the Holy Bible, First Kings (Chapter 3, verse 19): " and this woman's child died in the night because she overlaid it." Infanticide, or accidental infant death by negligence, was neither a criminal offense nor a moral issue during that era. It was not until 700 AD that the Roman Catholic Church imposed punishment for infanticide through overlying, although it was regarded as a relatively minor sin (Guntheroth, 1989). Throughout the fourteenth century, the

negligence leading to an infant death was regarded as a venial sin. By the sixteenth and seventeenth centuries, overlying was thought to be unintentional and measures were taken to prevent this accidental suffocation. These measures included the advent of a device called the *arcuccio,* which was an arch constructed of wood and iron that was placed over the infant's head during sleep. This arch is believed to have been used as late as the nineteenth century.

In the nineteenth century, the medical profession became actively interested in these sudden deaths in infants. By the late 1800's the prevailing theory as to the cause of death was an enlarged thymus pressing against the trachea. This was thought to diminish the airway and blood supply to the brain, or in some way to interfere with the functioning of the heart and lungs (Steele, 1980, cited in Guntheroth, 1989). The disorder was initially termed "thymica asthma" and later renamed "status thymico-lymphaticus". Unfortunately, the comparison of an enlarged thymus in infants who had died suddenly to a smaller thymus in control cases with a chronic disease was not valid. Infants with a chronic disease would be more likely to have an atrophied thymus, creating the false impression of an enlarged thymus in the sudden death victims.

Nearing the end of the nineteenth century, a revisited theory of suffocation became popular (Templeman, 1892). In an epidemiological study of 258 sudden infant deaths, Templeman found that there was a preponderance of cases among illegitimate infants and infants from the poorer classes. He concluded that these deaths involved ignorance, carelessness, and drunkenness of the caretaker, in combination with conditions of overcrowding. He added that these infant deaths

were not likely due to premeditated infanticide, but rather were caused by negligence deserving of prosecution. Templeman believed that enforcing child welfare laws would save infant lives. The incidence and epidemiology of these "suffocation" deaths are similar enough to SIDS to warrant reclassification of these cases (Hasselmeyer and Hunter, 1988).

In the mid twentieth century Werne and Garrow (1947, 1953) found reason to suspect a respiratory aliment behind the sudden infant deaths. Evidence of a recent upper respiratory infection was found in 33% of the infants, who had died suddenly and unexpectedly. Upon microscopic examination, they found pulmonary inflammation, and were able to grow postmortem cultures of bacteria. From this, they concluded that subtle respiratory infection was involved in the cause of death. Following publication of these articles, public pressure mounted for researchers to conduct more detailed investigations into these unexplained deaths.

The evolution of thought regarding the pathogenesis of SIDS has gone through major revisions emerging from many varied disciplines. By the 1960's, theories were offered following studies of behaviour, immunology, metabolism, respiration, cardiology, fetal and neonatal development, and neurology, to name only a few. For example, Parish et al. (1960) suggested that SIDS was an anaphylactic response to the regurgitation of cow's milk from the stomach during sleep. He presented an animal model using the guinea pig, exhibiting hypersensitivity to milk and enduring respiratory arrest, followed by a quick, struggle-free anaphylactic death. Although several epidemiological studies found that a greater proportion of SIDS victims are bottle fed than breast fed (Biering-

Sorensen et al., 1978), others studies have shown no difference in the immune response to cow's milk in SIDS victims (Gold and Godek., 1961; Coe and Peterson, 1963).

A related study found IgE antibodies to house dust mites in 37% of SIDS cases compared to 7% of controls (Turner et al., 1975). These authors proposed that this caused an anaphylactic death in SIDS victims. They also found increased frequency of antibodies to cow's milk and to aspergillus in SIDS when compared to control infants. However, subsequent authors were unable to replicate these findings (Mirchandani et al., 1984). No significant elevations of either total IgE or specific IgE to house dust, house dust mites (Dematophagoides farinae), mold (Alterarnia tenuis), or milk proteins was detected in SIDS victims when compared to controls.

Theories involving infections of both the mother and the postnatal infant have been postulated. Werne and Garrow (1947, 1953) reported that autopsy material from 67 of 124 SIDS cases showed microscopic changes indicative of infection. Adelson and Kinney (1956) found microscopic respiratory tract inflammation in 84% of the SIDS infants. Although there has been a long and varied history of ingenious and imaginative theories as to the cause of SIDS, most have not held up to persistent scrutiny.

At present, the cause of SIDS remains unknown. Most SIDS cases occur between 1 to 6 months of age with the peak age being around 2-4 months. Given this narrow age distribution, it appears in the most general terms as though SIDS involves dysfunction in development during a critical postnatal stage. The

developmental disorder alone is not enough to cause death, but rather produces susceptibility in an infant. Hypothetically, when these susceptible infants are exposed to an unknown trigger factor in the immediate environment, death ensues.

Evidence for susceptibility in SIDS victims includes numerous studies of sudden death in siblings. The risk of sudden death for a twin sibling of a SIDS victim is ten times greater than that of the general population (1% versus 0.1-0.2%, Beal, 1988). In fact, several twin pairs have been reported to die on the same day (Arsenault, 1980; Carpenter et al., 1977; Froggatt et al., 1971). The risk of sudden death in subsequent siblings of a SIDS victim is almost four times greater than in the general population (0.4% versus 0.1-0.2%, Guntheroth et al, 1990; Peterson et al., 1986; Irgens et al., 1988; Beal and Blundell, 1988). Therefore, twin death and sibling death are suggestive of a common developmental disorder, and perhaps a common environmental trigger factor at a vulnerable developmental period.

It is likely that the pathology involved in producing susceptible infants is multifactorial (Schwartz and Segantini, 1988). This, and the fact that autopsies of SIDS victims show subtle pathologies, which in and of themselves are not life threatening, makes the study of SIDS complex.

1.1.2. Epidemiology

SIDS is the leading cause of infant death (Schellscheidt et al., 1997), the incidence being 1-2 cases per 1000 live births. There is a time frame between 1 and 4 months of age, where SIDS is most prevalent (Kinney et al., 1992). At this time in development, many events and maturational changes are occurring. The

sleep-waking cycles are becoming established, the child's growth rate at this time is at a maximum, neuronal circuits are being elaborated and consolidated into their adult form (Kinney et al., 1992). A further report by Filiano and Kinney (1994) suggested three overlapping factors in the pathogenesis of SIDS: 1) vulnerability of the infant at a 2) critical developmental period in homeostatic control and 3) an exogenous stressor(s) or trigger factor. Due to the complexity of these developmental events, many developmental abnormalities could render the infant susceptible to SIDS.

Perhaps the most important epidemiologic finding in SIDS research is the increased incidence in infants who were placed to sleep in the prone position. The idea that sleep position is important was postulated as early as 1944 (Abramson, 1944). The author advised that the prone position be used with caution, as it could potentially lead to accidental suffocation. Studies on sleep position continued, but seemed to gain momentum after the 1985 observation that infants in Hong Kong, who traditionally slept in the supine position, had a relatively low incidence of SIDS (Davies, 1985).

Numerous studies have reported that infants lying in the prone position have a significantly higher risk of SIDS than infants lying in the supine position (Vandenplas et al., 1996; Kemp, 1996; Guntheroth and Splers, 1996; Oyen et al., 1997; Schellscheidt et al., 1997; Sawczenko and Fleming, 1996). Campaigns to encourage parents to place their infant in the supine position during sleep resulted in a subsequently decreased incidence of SIDS (Dwyer and Ponsonby, 1996). For example, the incidence of SIDS fell by 52% in one region of the United States, eight

months after a newspaper article advising parents against placing infants in the prone position during sleep. By contrast, in several other regions, which did not distribute this article, the incidence of SIDS increased by 3.4%. It is important to note that not all SIDS cases are found in the prone position. According to one recent study, 54 of 78 SIDS victims (69%) were found dead in the prone position. Only 20 of 100 of infants, dying of a known cause during sleep (20%), were prone at death (Schellscheidt et al., 1997). Overall, the strongest support for this causal association has been the decreased incidence of SIDS when infants are placed in the supine position (Mitchell, 1997). On an international level, the number of deaths has decreased by 1/3 since the "Back to Sleep" campaign in 1992 (Dwyer and Ponsonby, 1996).

Several hypotheses have arisen to explain the etiology of SIDS in the prone position. Many of these authors suggest that the prone position places the infant at greater risk of hypoxia. The association with hypoxia has been shown (Schellscheidt et al., 1997). This group found that placing infants to sleep in the supine position reduced the risk of hypoxia by 1/3 in susceptible infants. They suggest that this hypoxic environment is the trigger factor for susceptible infants.

A study by Bell et al. (1996) showed an additional finding with prone infants. Nasal swabs were taken after one hour of prone sleeping and after one hour of supine sleeping. Results revealed significantly higher bacterial counts in the prone position. Because approximately 25% of SIDS cases show microbiological and histological evidence of upper respiratory tract infection (i.e. respiratory syncytial virus), they could be more susceptible to the bacterial overgrowth in the prone

position (Krous, 1988). The toxins produced by these bacteria or the subsequent reduced nasal airway has been suggested to be an important trigger factor in SIDS (Krous, 1988).

Kinney et al. (1992) have reported abnormalities in brainstem areas controlling arousal and ventilatory response to hypercarbia and/or hypoxia. They suggest that proper functioning of these areas could be critical while sleeping in the prone position, if the infant is rebreathing carbon dioxide from a pocket of air formed between the infant's face and the mattress. Consistent with this hypothesis, Kemp (1996) produced an animal model of increased prevalence of face-down and face-into-bedding deaths, suggesting that rebreathing exhaled gases contributes to SIDS. He found that certain bedding materials are associated with a higher incidence of death. Specifically, sedated rabbits were placed face-down on ordinary bedding material, cushions filled with polystyrene beads, and sheepskins. Despite sedation, the animals responded vigorously to this insult and developed acidemia, progressive hypercarbia, and severe hypoxemia, frequently ending in death. It appears as though sleeping in the prone position provides some unknown stimulus which acts as a trigger factor in the etiology of SIDS.

There is an increased incidence of SIDS with maternal smoking, the risk being greater with the amount smoked (MacDorman et al, 1997; Schellscheidt et al., 1997). This could be associated with overall delayed development of the fetus during gestation, or to the toxicity of carbon monoxide from tobacco smoke (Blair, 1996). Postnatal exposure to smoking has also been implicated as being a risk factor (Hillman et al., 1997; Blair et al., 1996; Schellscheidt et al., 1997; review,

Golding, 1997). This may be a respiratory irritant which helps precipitate hypoxia or apnea associated with SIDS. Maternal drug abuse has been found to have an additional effect (Blair et al., 1996; Fares et al., 1997).

There appears to be a preponderance of SIDS deaths during the cold winter months (Swaczenko and Fleming, 1996; reviewed by Peterson et al., 1988). This is commonly thought to be due to over-bundling the infant, which could cause an increase in body temperature or cause an environment which would allow for suffocation (Kleenman et al., 1996). Alternative studies suggest that seasonal differences in circulating hormone levels during gestation may be the risk factor for the developing fetus (Raring, 1974; Bergman et al., 1970). By contrast, viral infections and nasal congestion due to common colds are more common in the winter months (Mitchell, 1989). The resulting irritation or partial occlusion of the respiratory tracts could act as a trigger factor in susceptible infants.

Epidemiological studies have also shown a 1.4 fold increase in the risk of SIDS for male infants (Mitchell and Stewart, 1997). It is unknown at present what factors may play a role in this distinction. Racial distinctions are also quite apparent. Numerous studies have shown a 1.7 to 4.0-fold increase in the incidence of SIDS in black infants when compared to white and hispanic infants, independent of any other factor such as low birth weight (MacDorman et al., 1997; Wegman, 1989; Black et al., 1986; Grether et al., 1989; Hunt, 1992). In North American native infants, the incidence of SIDS is 3.7 times greater than the general population, while Asian infants have been found to have the lowest incidence at approximately 0.4 deaths per 1000 live births (Davies, 1985).

Premature and low birth weight babies are at an increased risk of SIDS (Reid and Tervit, 1997; Grether and Schullman, 1989; Black et al., 1986; Yount et al, 1979). This could be due to underlying pathologies such as retarded CNS development, nutritional insufficiency, or maternal smoking.

Several studies identified low socio-economic status (Peterson et al., 1980), low weight gain (poor nutrition) of mother during pregnancy (Peterson et al., 1980), bottle fed infants (Mirchadani et al, 1984), mother's age less than 20 at first pregnancy (Hoffman et al., 1988), and anemia due to insufficient iron intake during pregnancy (Hoffman et al., 1988) as being risk factors.

1.1.3. Developmental Abnormalities and Postmortem findings

Although the definition of SIDS states that a complete autopsy reveals no pathology, some abnormalities are observed. These observations, however, are subtle, and are not considered to be consistent with having caused death (Valdes-Dapnea, 1975). Gross examination frequently reveals a frothy fluid from the mouth and nares, mild pulmonary inflammation with intrathoracic petechiae, and vascular congestion. Histolopathology includes hypertrophy of pulmonary arteriolar smooth muscle (Williams et al., 1979), right ventricular hypertrophy (Valdes-Dapena 1980), prolonged retention of periadrenal brown fat (Naeye, 1984; Valdes-Dapena et al., 1976), increased hematopoiesis (Gilbert-Barness et al., 1991), and reports of brainstem astrogliosis (Kinney et al., 1983; Naeye, 1976; Quattrochi et al., 1985; Takashima et al., 1978).

1.1.3.1. Respiratory Abnormalities

The most common observation in SIDS cases at routine autopsy is intrathoracic petechiae on the surfaces of the thymus, pleura, and pericardium. This finding implies respiratory obstruction at the time of death. An interesting study investigating petechiae in SIDS victims and in cases of lethal upper airway obstruction showed that petechiae were limited to the chest cavity in both groups (Krous and Jordan, 1984).

Numerous authors have demonstrated in animal models that hypoxia may be sufficient to cause these hemorrhages (Abu-Osbu et al., 1981; Kinney et al., 1992; Stowens, 1957; Handforth, 1959). For example, Campbell and Read (1980) showed that repeated exposure to hypoxia through obstruction in rabbits resulted in petechial hemorrhages in the lungs. Harrison (1991) reported an increase in mucous-secreting glands in the subglottis causing a reduction in the subglottic airway. This obstruction was also consistent with the presence of petechial hemorrhages.

One animal study, however, has shown that occlusion of the airway was not sufficient to produce petechiae consistently in animals (Guntheroth, et al., 1980). Thus, there remains some discrepancy as to the cause of these hemorrhages in SIDS.

It should be noted that, although there is general agreement that petechiae are frequently present in SIDS cases, there is evidence for considerable intra- and inter-observer variability (Byard and Krous, 1995). These investigators found that

there was a tendency for pathologists to overlook isolated or small numbers of inflammatory cells. A new histological technique to stain lung and thymus sections has been developed to better determine the number and size of petechiae, which are indicative of previous hypoxic episodes (Byard et al, 1997). This stain is specific for the hemosiderin-containing macrophages that are associated with these hemorrhages.

As previously mentioned, there are several other pathological findings that may give evidence of a respiratory involvement in the demise of SIDS victims. Investigators have found Increased pulmonary artery muscle mass in half of the SIDS cases when compared to controls (Naeye, 1973; Weiler and deHaardt, 1983). This could be caused by chronic hypoxia, which leads to pulmonary hypertension and possible thickening of vessels.

Histological studies of the carotid bodies, which are situated bilaterally at the bifurcations of the common carotid arteries, have reported both hypoplasia and hyperplasia in individual SIDS cases (Cole et al., 1979; Perrin et al., 1984). These abnormalities are important because of the role of the carotid bodies in regulating cardiovascular and respiratory functions. The carotid bodies serve as peripheral chemoreceptors and baroreceptors (Eyzaguirre and Zapata, 1984).

Chronic hypoximia also has an effect on the amount of brown fat (Teplitz and Lim, 1974). SIDS victims have been reported to have an increased percentage of periadrenal brown fat cells (Naeye, 1974), suggesting that a respiratory insult was present prior to death.

Increased hepatic erythropoiesis in SIDS has been reported in several

studies (Naeye 1974, Naeye et al., 1976, Valdes-Dapena et al., 1976). The liver does not contribute to erythropoiesis to a large extent under normal conditions, but increases in activity in response to chronic hypoxemia (Krous, 1988). This, again, implies respiratory pathology in SIDS.

Many premortem abnormalities in respiratory physiology have been observed in infants, who subsequently died of SIDS. Apnea of infancy is a common phenomenon, where an infant has short pauses in breathing of 20 seconds or longer (Brooks, 1982). Numerous physiological studies have shown that infants at high risk for SIDS have prolonged episodes of apnea (Brooks, 1982; Steinschneider, 1972; Guilleminault et al., 1978; Steinschneider and Rabuzzi, 1976; Shannon et al., 1977; Cornwell et al., 1978), and excessive perodic and short apnea (Steinschneider and Rabuzzi, 1976; Guilleminault et al., 1979; Kelly and Shannon, 1979). Additional evidence includes chronic tissue pathology consistent with hypoxia (Naeye, 1974; Naeye, 1976; Takashima et al., 1978). Measurements of postmortem brain pH and lactate concentrations have demonstrated that many SIDS victims have endured bouts of hypoxia prior to death (Butterworth and Tennant, 1989).

Recently, Hills et al (1997) have shown through broncho-alveolar lavage that SIDS victims had a higher total bile acid concentration in the lungs, when compared to controls. This abnormality in lung surfactant was suggested to be a causal factor in producing the increased apnea in SIDS. Hills et al. (1997) suggest that a measurement of the acid concentration in infants may have the potential to evaluate risk.

A study of the gasping reflex was investigated in neonatal, weaning and adult mice (Gershan et al., 1989) This group found that the infant mouse had a higher tolerance to hypoxia, which delayed the gasping reflex. This may be important in SIDS research because the longer an infant is in a state of hypoxia, the lower the ability to gasp (Hunt, 1992). Hunt (1992) noted that infants with apnea have diminished ventilatory responsiveness to hypercarbia and to hypoxia. If there is an underlying arousal or gasping deficiency, an infant prone to apneustic spells may be at a higher risk for SIDS.

There are many mechanisms that may cause apnea of infancy or prolonged hypoxia: seizure, laryngeal chemoreception failure, upper airway or tracheal obstruction, failure of automatic ventilation during sleep, cardiac arrhythmia, congenital heart disease, sepis and meningitis, hypocalcemia, hypoglycemia, respiratory infection, anemia, and CNS tumors (Harper et al., 1988). An interesting theory speculates that seizure discharge, which is enhanced during sleep, may be occurring in brainstem and pontine regions controlling respiration and cardiac functioning (Harper et al., 1988). Respiratory and cardiac failures from such epileptic episodes would be sudden and silent. In addition, the upper thoracic petechiae, seen in the majority of SIDS cases, could be the result of extended inspiration against excessive seizure-induced laryngeal muscle activation (Harper et al., 1988). Of course, not all of these proposed mechanisms apply to the potential cause of apnea in all SIDS cases. However, many of these proposed mechanisms would be undetectable after minimal postmortem delay, which makes it difficult to determine the potential causes.

The most prevalent postmortem finding of intrathoracic petechiae suggests that apnea does play a role in the pathogenesis of SIDS in many individual cases. As mentioned above, breathing against a closed airway can produce changes in the intrathoracic pressure and subsequent microvasculature rupture. Finding specific causes would aid in the identification of susceptible infants.

1.1.3.2. Cardiac abnormalities

Many theories have proposed that death in SIDS cases is the result of a lethal arrhythmic episode. Subtle cardiac abnormalities have been documented at postmortem investigations, but these are seldom sufficient to cause death.

During development, many changes are occurring in the heart. An interesting change is the transformation of the atrioventricular node and the penetrating bundle from the neonate to the adult. At birth the node is "shaggy" in appearance (Ho and Anderson, 1988). The transformation process to the smoothly outlined configuration, characteristic of the adult, involves active resorption and necrosis (Anderson et al, 1970). Some studies have found a maturational delay in this molding process in SIDS (Anderson et al., 1970; James, 1968). However, other authors have reported a similar maturational delay in a small proportion of control cases (Lie et al., 1976). It is controversial as to whether or not the failure of this resorptive process could be capable of producing cardiovascular instability sufficient to cause death.

Naeye et al. (1976) reported right ventricular hypertrophy in SIDS cases, which they indicated was likely caused by chronic hypoxia. This finding, however,

was not subsequently replicated (Valdes-Dapena et al., 1980). Myocarditis has been reported to cause SIDS (De Sa, 1985; Jankus, 1976). The cases that were found to have myocarditis were improperly diagnosed as SIDS and does not represent the sudden infant death syndrome (Huang, 1983).

Although little pathology is found in postmortem cardiac tissue, several studies implicate abnormalities in cardiorespiratory physiology. Premortem studies of infants, who subsequently died of SIDS, had higher heart rates and less heart-rate variability (Southall and Talbert, 1988; Valimaki et al., 1988; Leistner et al., 1980), increased tachycardia (Valimaki et al., 1988 ; Schechtman et al., 1990), depressed hypercarbic ventilatory responses, impaired regulation of alveolar ventilation (Shannon and Kelly, 1977), and impaired cardiorespiratory coupling.

Because parallel abnormalities exist in respiratory and cardiac physiology in SIDS cases, the mechanism of death could conceivably involve impaired central nervous system control by brainstem neurons, which regulate the physiology in both systems.

1.1.4. Central nervous system abnormalities

In SIDS victims, brain weight has been shown to be significantly increased when compared to non-SIDS controls (Shaw et al., 1989; Kinney et al., 1991; O'Kusky and Norman, 1992; O'Kusky et al., 1995). In addition, the rate of increase in brain weight during postnatal development was found to be greater in SIDS (O'Kusky and Norman, 1992). Body weight, however, did not differ between the two groups (O'Kusky et al., 1995). Consistent with a larger brain weight, several

regional volumes in the brainstem have been found to be significantly increased in SIDS. These areas include the pons (33%), nucleus pontis (38%), and medulla (19%), but not the cervical spinal cord (O'Kusky et al., 1995). Not only did not mean volumes differ, but the rate of volume increase was significantly higher in the pons (56%) and the nucleus pontis (83%) from birth to one year of age (O'Kusky et al., 1995).

Specific brainstem nuclei have also been investigated. Consistent with an increased brain weight, the mean volume of the hypoglossal nucleus was found to be significantly larger in SIDS than in control infants, and had a faster rate of growth (79%) during development (O'Kusky and Norman, 1992). Upon further investigation, O'Kusky and Norman (1992) found that this volume increase was not due to an increase in neuron number, but due to an increase in neuropil surrounding the neurons. This corresponds well with the later finding of an increased density of synapses in the reticular formation (38%), and an increased total number of synapses (61%) in the hypoglossal nucleus (O'Kusky and Norman, 1994; O'Kusky and Norman, 1995) Other authors have reported similar results for estimates of the density of dendritic spines in several brainstem nuclei. The areas that have shown a significant increase in the density of dendritic spines include the ventrolateral medulla (Takashima et al., 1994), reticular formation (Takashima and Becker, 1985; Takashima et al., 1994), dorsal vagal nucleus (Takashima et al., 1994; Becker and Zhang, 1996), the tractus solitarius (Becker and Zhang, 1996; Quattrochi et al., 1985), the nucleus ambiguus (Takashima and Becker, 1985; Takashima et al., 1994; Quattrochi et al., 1985), hypoglossal nucleus, nucleus of

the lateral lemniscus, spinal trigeminal nucleus, nucleus gigantocellularis, nucleus pontis caudalis (Quattrochi et al., 1985). Spine density of catecholaminergic neurons were also found to be increased in the ventrolateral medulla, reticular formation, dorsal motor nucleus of the vagus, and the nucleus ambiguus (Takashima and Becker, 1991). It is possible that the increase in nuclear volume found is due predominantly to the increased connectivity present in a larger volume of neuropil. This excessive amount of synaptic connectivity may contribute to the cardiorespiratory instability characteristic of infants susceptible to SIDS.

Morphometric analyses of the arcuate nucleus of the medulla have reported both hyperplasia (Gilson et al., 1994) and hypoplasia (Filiano and Kinney, 1992). The arcuate nucleus is situated on the ventral surface of the medulla and, based on homologous brainstem regions in animals, is thought to function in chemosensitivity, ventilation, autonomic function, and arousal. Animal studies have shown that ablating this area of brainstem produces apnea (Millhorn and Eldridge, 1986; Nattie in Haddad et al., 1991), and local application of glycine, glutamate, opioids, cholinergic agents, and somatostatin alters ventilation and cardiopressor reflexes (Millhorn and Eldridge, 1986; Chen et al., 1990).

Several neurochemical anomalies have been reported in the central nervous system of SIDS victims, which may help explain the morphological findings. For example, the capacity to synthesize adrenaline was decreased in several areas of the medulla, including the respiratory centers (Denoroy et al., 1987). Consistent with these results, Ozand and Tildon (1983) found that Dopamine-β-Hydroxylase activity, a catecholamine enzyme, was decreased and tyrosine hydroxylase activity

was increased in the hypothalamus, putamen, and caudate nucleus of SIDS victims. They suggested that this decreased enzyme activity is a result of hypoxia. They also speculate that malnutrition or exposure to ethanol may be involved in these abnormalities.

Substance P, which is a powerful stimulant of respiration, was found to be significantly elevated in the medulla of SIDS when compared to controls (Bergstrom et al., 1984) and substance P-immunoreactive nerve fibers were increased in the pons (Takashima et al., 1994). Levels of enkephalins in SIDS victims, however, did not differ from controls (Bergstrom et al., 1984). Substance P and enkephalins have opposite effects on respiration, and together act to regulate respiratory functioning.

Astrogliosis has been reported in selected brainstem regions in SIDS victims (Kinney et al., 1983; Naeye et al., 1976; Quattrochi et al., 1985; Takashima et al., 1978; Takashima and Becker, 1985, Becker and Takashima, 1985). However, this finding is controversial, due to the many investigations that have found no astrogliosis in selected regions of the brainstem, including morphometric analyses by our lab (Pamphlett and Treloar, 1996; Ochmichen et al., 1989; Reske-Nielson, et al., 1987; Pearson and Brandeis, 1983; Ambler, et al., 1981, O'Kusky and Norman, 1992). Astrogliosis in the brains of some SIDS victims may be indicative of excessively prolonged hypoxic insult prior to death.

Delayed CNS myelination has been implicated in SIDS (Becker et al., 1993; Sachis et al., 1981). Sachis et al. found a lower number of small myelinated vagal fibers in the vagus nerve, with a gradual increase with postconceptional age. These small myelinated fibers innervate "irritant" receptors of the trachea and contribute

to respiration patterning. A decrease in these fibers could be a potential cause (or effect) of the cardiorespiratory pathologies indicated for SIDS with the mechanism of death involving rapid shallow breathing in response to an irritant. In 1993, Becker et al. substantiated these findings. They showed more small-diameter myelinated fibers in SIDS with reduced thickness in the myelin sheath, and significantly smaller axonal diameters. As well, Kinney et al. (1991) reported hypomyelination in multiple regions of the brainstem in SIDS victims when compared to age matched controls.

Postmortem measurements of the pineal glands revealed smaller glands in SIDS cases than in the control cases (Sparks and Hunsaker, 1988). As well, cerebrospinal fluid levels of melatonin were found to be correspondingly lower in SIDS (Sturner et al., 1990). Melatonin is thought to be involved in REM sleep (Maurizi, 1985), and decreased amounts of a melatonin precursor would cause a decreased amount of REM sleep. Because REM sleep has been said to protect an infant from sudden infant death (Maurizi, 1985), decreased melatonin could conceivably be involved in the etiology of SIDS.

The consensus of most morphological studies of the brain in SIDS is that there is an overgrowth phenomenon occurring during late prenatal and early postnatal development. Brain weight and the rate of increase in brain weight during postnatal development are significantly greater in SIDS. The volumes of the medulla, pons and specific brainstem nuclei are larger in SIDS, although individual nuclei contain the normal complement of neurons. Increased numbers of dendritic spines and synapses are consistent with an increased complexity of axonal circuitry in SIDS when compared to age-matched controls. Many of the areas found

to be abnormal are germane to respiratory physiology, cardiac physiology, and arousal. Any developmental disorder of circuit formation and synaptogenesis in these brainstem nuclei could conceivably produce the cardiorespiratory instability involved in the pathogenesis of SIDS.

1.2. Synaptogenesis

It has been shown through morphometric and stereological analyses that synaptogenesis occurs in two distinct phases. In the initial progressive phase, there is a significant increase in the numerical density (N_V, synapses per unit volume) and total number of synapses to peak values at some characteristic age. During the regressive phase, there is a gradual decrease in the N_V and total number of synapses to values characteristic of the young adult. This pattern of development has been termed synaptic overshoot and has been reported, for example, in the cerebral cortex of rat (Aghajanian and Bloom, 1967; Markus and Petit, 1987), cat (Cragg, 1975; Winfield, 1981; O'Kusky, 1985), monkey (O'Kusky and Colonnier, 1982; Rakic et al., 1986) and man (Huttenlocher, 1979; Huttenlocher et al., 1982; O'Kusky et al., 1996). The time course of this synapse elimination, however, differs between species. For example, synaptic density has been found to reach a peak value in the cat visual cortex at around postnatal day 70, followed by a gradual decrease to levels characteristic of the young adult (Winfield, 1981). The peak age for synaptic density occurs at 4-6 months in the monkey (Rakic et al., 1986). and as late as 8 months in humans (Huttenlocher et al., 1982). Estimates of the magnitude by which peak synaptic densities exceed those of the young adult have

been reported to vary from 30-50% in most studies. It has also been shown that there is postnatal overshoot in the cortical volume, with a subsequent decrease in cortical thickness and surface area (Zilles, 1978; O'Kusky and Colonnier, 1982; Sauer et al., 1983). Parallel to synapse development, dendritic spines have also been found to undergo overshoot and subsequent elimination (Boothe et al., 1979; Michel and Garey, 1984). Although a substantial number of axon terminals and synaptic contacts are eliminated from the cerebral cortex during postnatal development, the parent neurons giving rise to these axons and synapses have not been identified.

Additional examples of axon remodelling, where both the location of the parent cell body and the target of the efferent axon are known, have been demonstrated in corticofugal and descending supraspinal pathways. This remodelling results in the complete elimination of some pathways and in a substantial reorganization of others. The neuronal cell bodies in the cerebral cortex which give rise to corticospinal axons can be identified by the retrograde transport of certain markers (fluorescent dyes, horseradish peroxidase), when these are injected into the spinal cord of experimental animals. When injections are made in the neonatal rat, labelled neurons are located in layer V throughout the neocortex, including the occipital visual cortex. When similar injections are made in young adults, corticospinal neurons are located in layer V in a discrete region of the frontoparietal cortex. Several studies have demonstrated a major redistribution of corticospinal neurons with complete elimination of the pathway originating in the occipital cortex (Stanfield et al., 1982; Bates and Killackey, 1984; Stanfield and

O'Leary, 1985). This remodelling of corticospinal axons results from the elimination of axonal collaterals rather than from the death of the parent cell body. This remodelling has been shown in other areas, such as the corticotectal pathway (Ivy and Killackey, 1981), callosal projections between the two cerebral hemispheres (Wise and Jones, 1976; Innocenti et al., 1977; Innocenti and Caminiti, 1980; Feng and Brugge, 1983), thalamocortical projection (LeVay et al., 1978) and projections from the locus coeruleus to the spinal cord (Chen and Stanfield, 1987).

The mechanism of synapse elimination remains unknown. It is apparent that the process does not require the death of the parent cell body. Many studies focus on the elimination process in the neuromuscular junction. Initially, motor axons co-innervate muscle fibers. Elimination of these connections continue until the muscle fiber is singly innervated (Coleman and Lichtman, 1993). This mechanism is thought to involve competition for the uptake of neurotrophic factors from the postsynaptic target site (reviewed by Coleman and Lichtman, 1992). This mechanism, however, may or may not describe elimination in other areas of the central nervous system. Activity of the synapse is also thought to play a role in synapse elimination. This is best shown by the refinement of the retinotectal projection. During development, the retinal ganglion cells send axons to the optic tectum in lower vertebrates, and to the lateral geniculate nucleus of the thalamus in mammals. These axons form a coarse topographical map that needs refinement by elimination of extraneous projections and the synapses that they form to achieve sufficient resolution of fine details in the visual field. Several investigators have shown that blocking activity in this system prevents this fine-tuning (Fawcette and

O'Leary, 1985; Schmidt and Edwards, 1983; Meyer, 1982; Kobayadhi et al., 1990).

Synapse elimination appears to occur in all areas of brain. The persistence of dendritic spines and the increased total number of synapses found in the medulla and pons of SIDS cases (O'Kusky and Norman, 1995; O'Kusky and Norman, 1994; Takashima et al., 1994; Takashima and Becker, 1985; Becker and Zhang, 1996) can be interpreted as reflecting either an accelerated progressive phase of synaptogenesis, or a failure to eliminate normally transient axonal projections. It would be tempting to suggest that a failure to eliminate the normally extraneous synaptic contacts in selected brainstem nuclei responsible for controlling cardiorespiratory physiology and arousal would create susceptibility to SIDS due to improper functioning of these vital processes.

1.3.Dorsal motor nucleus of the vagus and the control of breathing

Because recent research implicates a cardiorespiratory mechanism in the pathogenesis of SIDS, a morphometric investigation of the dorsal motor nucleus of the vagus (DMV) may provide insight into the etiology of this disorder.

1.3.1. Neurobiology of the DMV

The DMV is bilaterally located in the medulla, along the floor of the IVth ventricle for most of its course. In the caudal portion of the medulla, the DMV is located lateral to the central canal, dorsal to the hypoglossal nucleus and ventral to the nucleus of the solitary tract . More rostrally it is located along the floor of the forth ventricle, medial to the sulcus limitans (see Fig. 2.1), situated lateral to the

hypoglossal nucleus and medial to the nucleus of the solitary tract. The dorsal motor nucleus of the vagus is comprised of medium sized neurons, which are preganglionic parasympathetic motor neurons, and small non-motor neurons (Sturrock, 1990; Aldskogius, 1978; Laiwand et al., 1987). The motor neurons have substantial amounts of Nissl substance and are clearly distinguishable from the non-motor neurons by light microscopy. The non-motor neurons are pale, with little to no Nissl substance.

The DMV is considered to be the principle parasympathetic nucleus of the brain. Axons from the DMV in combination with axons originating from the nucleus ambiguus comprise the vagus nerve. The DMV supplies the majority of the general visceral efferent fibers in the vagus nerve (Becker et al., 1993). The preganglionic neurons supply the thoracic and abdominal viscera, excluding the extrathoracic trachea (Kalia and Mesulam, 1980), with the majority of the fibers (~90%) supplying the stomach (Shapiro and Miselis, 1985). Numerous authors have reported that there is innervation of the heart and that the DMV has a role in the autonomic control of the heart (reviewed by Levy and Martin, 1984; Standish et al., 1995; Jansen and Loewy, 1994). For example, a convincing tract tracing study by Standish et al. (1994) showed that the DMV and NA extensively innervate cardiac ventricular sites, including the apex. This investigating group showed that it has profound effects on cardiac function, including the beat-to-beat regulation of cardiac rate. Previous studies, which have used various physiological tract tracing and denervation techniques, have found similar results (Calaresu and Cottle, 1977). In contrast, however, studies using horseradish peroxidase tracing techniques have

suggested a minor role for the DMV in heart functioning (Izzo et al., 1993; Escardo et al., 1991).

As well as having an influence in the functioning of the heart, the DMV is also involved in respiration (Lawson et al., 1992). Specifically, it contains neurons that are involved in the initiation and maintenance of inspiration. These neurons send efferents to the respiratory neurons in the nucleus of the tractus solitarius, which in turn innervate the phrenic nucleus, which in turn stimulates contraction of the diaphragm.

Afferents to the DMV include the nucleus ambiguus, hypothalamus, periaquiductal grey, and the amigdala (Kinney et al., 1992). The neurotransmitters and neuromodulators considered to be important in the DMV include thyrotropin releasing hormone (Hornby et al., 1989; McTigue et al. 1992), oxytocin (Rogers and Herman, 1987), serotonin (Hornby et al., 1990), γ-aminobutyric acid (Travagli et al., 1991), and L-glutamic acid (Travagli et al., 1991). Substance P may have a role in the functioning of the DMV (Obonai et al., 1996). It has been shown to enhance the effect of serotonin by depolarizing the membranes of the respiratory neurons (Takashima et al., 1994). Because the DMV has roles in the control of breathing and in the autonomic control of breathing, the function of the DMV, is of great importance to the study of cardiorespiratory abnormalities in sudden infant death syndrome, and will be elaborated in further detail.

1.3.2. Regulation of respiration in infants

The control of breathing is not fully understood, especially in that of a

neonate or infant. Historically, the respiratory center was thought to be a distinct locus in the brainstem. It is now well established that there is an intricate integration of neuronal pathways in various areas of the brainstem and spinal cord, as well as higher cortical influences (Beckerman et al., 1992).

In 1922, Lumsden postulated three levels of organization to describe the brainstem respiratory centers. The first center was termed the "pneumotaxic center", which was found to discharge rhythmically to produce the normal respiratory rhythm. This denotes the rostral 2-3 mm of the pons. The "apneustic center", located in the lower pons, is responsible for tonic facilitation of inspiration, but is overridden by the actions of the pneumotaxic center. The "gasping center" is located in the medulla and is the primitive rhythmic center for breathing. Its actions can be overridden by both the pneumotaxic and the apneustic centers of the brain.

At the neuronal level, it is now known that there are three major brainstem complexes containing the neurons that initiate and maintain respiration. The "dorsal respiratory group" contains the dorsal motor nucleus of the vagus and the nucleus of the tractus solitarius. These neurons are largely responsible for initiating inspiration. The "ventral respiratory group" consists of the nucleus ambiguus, the nucleus retoambiguus, and the surrounding central reticular nucleus of the medulla, and contains neurons involved in expiration. The third group of neurons is termed the "pontine respiratory group", and is located in the caudal pons, including the medial and lateral parabrachialis nuclei and the Kolliker-Fuse nucleus. The function of this group includes the tonic facilitation of inspiration.

Breathing ultimately relies on a triphasic rhythmic stimulation of the

diaphragm and the intercostal muscles (Richter and Ballantyne, 1983). The three phases of this rhythm generation include inspiration, postinspiration, and expiration. Initiation of inspiration involves integration of the dorsal respiratory group with sensory input from chemosensors in the ventral medulla, peripheral chemosensors in the carotid and aortic bodies, barosensors in the carotid sinuses, and stretch, mechano-, and irritant receptors in the lungs and upper airways (Kinney et al., 1992). The major drive is from chemoreceptor stimulation of the dorsal respiratory group neurons in response to changes in pH, pCO_2, and PO_2 levels. Higher levels of CO_2 are mediated by changes in H^+ concentration (Henderson-Smart and Cohen, 1988) and cause direct bronchiolar dilation, as well as stimulation of the carotid bodies. PO_2 can directly affect the microvasculature by causing dilation or constriction of the capillaries, as well as mediate by detection from chemoreceptors to illicit stimulation of the dorsal center. This effect has been found to be more pronounced in newborns (Henderson-Smart and Cohen, 1988).

On a neuronal level, inspiration involves the depolarization of inspiratory neurons in the dorsal respiratory group, which stimulates the phrenic nerve . These nerves originate in the spinal cord at the levels of the neck and thorax and cause contraction of the diaphragm and the external intercostals. Contraction of the diaphragm causes it to flatten, pulling the lungs into a more expanded shape, and the consequent negative intrapulmonary pressure draws air into the lungs. Lung stretch receptors stimulate postinspiratory neurons, which are located in the ventral respiratory nuclei (Lawson et al., 1992), the Botzinger complex (Richter et al., 1986), and motor neurons of the cranial nerves which innervate upper respiratory

airways such as the hypoglossal and glossopharyngeal motor neurons. These postinspiratory neurons depolarize immediately after inspiration to prevent the over expansion of the lungs and the reactivation of the inspiratory neurons until expiration has occurred (Lawson et al., 1992).

For expiration, expiratory neurons in the ventral respiratory group depolarize and cause the diaphragm and the external intercostals to relax and passively allow elastic recoil of the lungs. Active expiration during high drive states, however, occurs by innervation of the expiratory intercostals and abdominal muscles. Breathing patterns are influenced by this contribution to the termination of inspiration, This ventral respiratory center is also involved in the reception of information from the lung stretch receptors so as to not allow collapse of the lungs.

As an aside, respiration has been found to be closely linked with many cortical functions and physiological activities including metabolism, speech, temperature regulation, fluid and acid-base balance and thoracoabdominal expulsive acts (Beckerman et al., 1992). For example, the hypothalamus influences the rate of respiration. The hypothalamic regions active in temperature regulation can promote heat loss through additional respiratory evaporation. This may be an important factor relevant to SIDS because most SIDS victims, when found, are wet from perspiration and tend to be overbundled (Guntheroth, 1989). If SIDS is due to a respiratory disorder, this increased temperature may be the trigger factor that precipitates a lethal response.

1.3.3. Breathing in the fetus, neonate and infant

Respiratory activity begins during early gestation. This "fetal breathing" was first reported by Dawes et al. (1970). This group found that regular, intermittent breathing movements occurred in fetal sheep during REM sleep. Although these intermittent breathing movements are not responsible for gas exchange, it is thought that they may be important to the maturation and postnatal functioning of the lungs (Rigotto et al., 1986; Fewell et al., 1983). Fluid is obstructed during inspiration in fetal breathing at the level of the larynx and hypopharynx (Johnston et al., 1986). As well as providing no gas exchange, fetal breathing differs from air breathing in that it is not stimulated by hypoxia or hypercarbia (Johnston, 1993). In fact, there is a hypoxic inhibition of breathing controlled by lateral pontine sites (Johnston, 1993). These sites override the peripheral chemoreceptors unless the hypoxia is severe. In such case, the inspiratory obstruction is lost and meconium is aspirated into the lungs (Hooper and Harding, 1990). Hypercapnia, a more powerful stimulator of fetal breathing (Boddy et al., 1974), does not cause the upper airway to dilate until birth (England et al., 1982) disallowing aspiration of meconium. This inhibition is due to insufficient coordination of inspiratory upper airway and diaphragmatic action during gestation (England et al., 1982). Coordination is complete during the transition to air breathing allowing dilation of the upper airway and inspiration to occur at sufficient hypercapnia.

At birth, breathing becomes continuous and serves the purpose of gas exchange. The mechanisms involved in this change are unknown, although it is thought that it may be due to a chemical or hormonal trigger (Rigatto, 1992). If the umbilical cord is left intact and the oxygen level remains adequate, the response

postpartum is still that of breathing, increased arousal, metabolic rate, cardiac output, and heart rate (Johnson and Andrews, 1992). These responses are initiated due to the cooling of the fetus, which causes TSH and TRH to increase. These hormones activate the "non-shivering thermogenesis", which is inhibited throughout gestation (Sack et al., 1976).

Respiration in the neonate is quite irregular with high breath-to-breath variability (Rigatto and Brady 1972a; Rigotto and Brady 1972b). Also, apnea and periodic breathing during both REM and quiet sleep are frequent at this stage (Gabriel et al., 1976; Rigatto and Brady 1972a; Rigotto and Brady 1972b). This pattern subsides after approximately 44 postconceptional weeks, which is consistent with the prevalent time period of SIDS.

Neonates, infants and adults differ from each other in their duration of sleep, which is an important consideration for differences in respiration. Neonates spend a majority of their time (90%) in a sleep state, infants about half, and adults, one third. Respiration during a sleep state is different than that during wakefulness. This is due to the changes in homeostatic control systems. During sleep, the cortical override to apnea and periodic breathing is suppressed. Because of this, periodic breathing and apnea are more prevalent during sleep (Glotzbach et al., 1992). Both respiratory rate and heart rate are higher in REM than in NREM, but lower than that during wakefulness (Curzi-Dascalova et al., 1983). The fact that SIDS victims, prior to death, had longer durations of both REM and NREM sleep (for review see Kinney et al., 1992) may have been a factor affecting their potential respiratory control disorder.

In infants, respiration depends on airways allowing sufficient airflow, muscles that power airflow, lungs that allow gas exchange, and proper circulation to transport the gases. Given the fact that breathing is both autonomic and voluntary, and that there is such a diversity of interrelated functions during both wakefulness and sleep, it is not hard to imagine a developmental abnormality occurring.

1.3.4. Relevance of the DMV to SIDS

In infants susceptible to SIDS, a developmental disorder of brainstem neurons, which are involved in the control of cardiac physiology, respiratory physiology, and cardiorespiratory coupling, most likely contributes to their cardiorespiratory instability. Disordered development of the nucleus ambiguus and surrounding central medullary reticular nucleus (i.e. ventral respiratory group neurons) has been documented previously (O'Kusky and Norman, 1994). It is likely that neurons of the dorsal respiratory group in the DMV are also involved. The overgrowth of the brainstem and the presumptive failure to eliminate normally extraneous synapses could conceivably result from the increased expression of a neuronal growth factor during late prenatal and early postnatal development in SIDS cases. The determination and characterization of this growth factor would permit the identification of infants at risk.

1.4. Insulin-like growth factor-I in the CNS

A likely candidate for a neuronal growth factor that would produce overgrowth of the brainstem and prevent the normal elimination of synapses from

brainstem neurons during late prenatal and early postnatal development would be insulin-like growth factor I (IGF-I). Insulin-like growth factor-I (IGF-I) and insulin-like growth factor-II (IGF-II) are part of the insulin gene family. Historically it was thought that IGF-I acted in an endocrine manner, being secreted by the liver to influence growth and development. Although this holds true, it is now known that IGF-I is an anabolic peptide, which is expressed in most tissues from early embryonic development (for review, see D'Ercole et al., 1996) and functions in an endocrine, autocrine and paracrine manner (LeRoith et al., 1993). After birth, IGF-I expression increases to a peak, then decreases to levels characteristic of the adult. The developmental time period and amount of IGF-I expression is specific for each tissue, and is regulated by growth hormone (GH) which influences upregulation of IGF-I in tissues undergoing proliferation and/or neurite outgrowth (D'Ercole, 1996). It has been well documented that IGF-I can stimulate cell proliferation, differentiation, and neurite outgrowth in cell culture studies (for review see D'Ercole, 1996; Bondy, 1991). Observations of transgenic mice deficient in IGF-I show that they are born growth retarded by 40-45% (Liu et al., 1993). Transgenic mice deficient in IGF-I receptor are growth retarded during prenatal development and die in the early neonatal period (Liu et al., 1993). Transgenic mice that overexpress IGF-I present unique results in each tissue. Specific effects on the central nervous system are discussed in detail below.

The actions of IGF-I are regulated by two cell surface receptors, designated type I and type II IGF receptor, and by IGF-binding proteins. The α-subunit of the type I receptor responds to IGF-I by autophosphorylation of its β-subunit, thereby

initiating a cascade of phosphorylation reactions of a series of intracellular messengers (LeRoith et al., 1995). The type II IGF receptor is identical to the cation-independent, mannose-6-phosphate moieties and IGF-II to lysosomes for degradation. There is little convincing evidence that IGF interaction with this receptor signals IGF growth-promoting activity. The binding proteins, of which there are six identified (Shimasaki and Ling, 1992) modulate IGF actions by inhibition and augmentation with the cell surface receptor.

Growth and development of the central nervous system is thought to be influenced by IGF-I (Ishii, 1993). For example, transient IGF-I gene expression is quite pronounced in the cerebral cortex. Bondy (1991), found that the cerebral cortex had the highest intensity of IGF-I gene expression of any brain region postnatally. Investigations of transgenic mice overexpressing IGF-I are consistent with this finding. Ye et al., (1995), reported that the cerebral cortex in the transgenic mice was the most overgrown area relative to the control littermate mice.

Numerous studies have demonstrated a role for IGF-I in the development of the cerebellum (D'Ercole, 1996). It is thought that here, IGF-I is expressed predominantly by Purkinje cells and is both autocrine and paracrine. The fact that IGF-I is located throughout the circulatory system and can readily cross the blood brain barrier suggests an endocrine mechanism as well, but it is not known to what extent this extraneous source affects growth of the central nervous system. It has been found that cerebelli in transgenic mice, which overexpress IGF-I, are twice the size of the cerebelli of littermate controls at postnatal day 50 (Ye et al., 1996). Consistent with the findings that implicate IGF-I as a mitogen, the spatiotemporal

expression correlates perfectly with the timing of cerebellar development (D'Ercole et al., 1996). Because the neural packing density and the DNA content is also increased in these transgenic mice, it is thought that the regulatory role in the cerebellum is through proliferation of cells and increased survival from the natural occurring neuron death.

Transient IGF-I gene expression has been reported in discrete brainstem nuclei with peak gene expression occurring at markedly different postnatal ages in individual nuclei (Bondy, 1991; Bondy et al., 1992). IGF-I mRNA was found predominantly in long-axon projection neurons with peak expression occurring relatively late in development, at a stage characterized by the maturation of dendrites and the progressive phase of synaptogenesis. IGF-I gene expression was greatest in specific groups of functionally related sensory and cerebellar projection neurons (Bondy, 1991). IGF type I receptor gene expression was shown to be uniform in the brainstem, in all neuroepithelial cell lineages, with regional variation reflecting primarily differences in neuronal density (Bondy et al., 1992). The highest levels of IGF type I receptor gene expression were observed in neurons with correspondingly high levels of IGF-I mRNA (Bondy et al, 1992). In transgenic mice, which overexpress IGF-I, significant increases in brainstem weight have been reported (Ye et al., 1996).

The IGFs appear to be involved in the control of synaptogenesis in the peripheral nervous system. The IGF-II gene is expressed in muscle at levels closely correlated with the development of motor synapses (Ishii, 1989). The elimination of multiple neuromuscular synapses from birth to day 15 in the rat is paralleled by a

down-regulation of IGF-II (Ishii, 1989) and IGF-I (Glazner and Ishii, 1989) mRNA levels. The presynaptic blockade of neuromuscular synapses with botulinum toxin prevents the postnatal decline in IGF mRNA in muscle and simultaneously reduces the rate of synapse elimination (Brown et al., 1981; Thomson et al, 1979). IGF-I mRNA has been shown to be focally localized in the peripheral target fields of trigeminal and sympathetic nerves during their time of innervation (Bondy and Chin, 1991; Bondy, 1990).

There is increasing evidence that IGF is involved in wound repair, axon sprouting, and synapse regeneration. Subsequent application of recombinant IGF-I after experimental lesion of the sciatic nerve in an adult rat increases the length of axon regeneration (Kanje et al., 1989). Also, studies have shown that mRNA for both IGF-I and IGF-II increased following hypoxic-ischemic injury in rat brain (Beilhartz et al., 1995; Klempt et al., 1992).

It has been postulated that SIDS results from a failure to eliminate normally transient axonal projections and their associated synapses in brainstem nuclei. In the peripheral nervous system IGF-I has been shown to control both the progressive and regressive phases of neuromuscular synaptogenesis. If IGF-I has a similar function in the central nervous system, then its overexpression during late prenatal and early postnatal development would likely produce developmental disorders of the brainstem similar to those documented in the pathogenesis of SIDS. The exogenous application of IGF-I to the developing brainstem of experimental animals could conceivably be used to produce an animal model of SIDS. In human neonates, elevated levels of IGF-I in placenta, umbilical cord,

circulating blood, or cerebrospinal fluid might provide the basis for a diagnostic test to identify susceptible infants.

1.5. Aims and objectives of the study

The present study was designed to investigate developmental abnormalities in the dorsal motor nucleus of the vagus (DMV) in sudden infant death syndrome:

1) In the first experiment, presented in chapter 2, morphometric analyses were conducted on autopsy material from the brainstem of SIDS cases and age-matched control cases without neurological disease. Postnatal changes in the total volume of the DMV were compared in SIDS cases and controls. Furthermore, postnatal changes in the numerical density and total number of neurons, as well as the mean neuronal profile areas, were determined in these groups. Given the previous studies demonstrating overgrowth of various brainstem nuclei in SIDS, it was expected that in the SIDS cases 1) the nuclear volume of the DMV would be significantly greater, 2) the numerical densities of neurons would be significantly decreased 3) the total number of neurons would remain unchanged, and 4) the mean neuronal profile areas would be significantly larger.

2) In the second experiment, presented in chapter 3, morphometric analyses were performed on the DMV and hypoglossal nucleus in transgenic mice, which overexpress IGF-I postnatally, and in normal littermate controls on postnatal day 35. The total volumes of the DMV and hypoglossal nucleus were compared in transgenic mice and normal controls. In addition, the numerical density and total

number of neurons, as well as the mean neuronal profile areas, were determined in these groups. Given that IGF-I has been shown to promote neuronal survival and growth, it was expected that in the transgenic mice 1) nuclear volumes would be significantly greater, 2) the numerical densities of neurons would be either increased or unchanged, 3) the total number of neurons would be significantly greater, and 4) the mean neuronal profile areas would be significantly larger in both nuclei. The purpose of this analysis was to investigate the extent to which elevated IGF-I in the brainstem during early postnatal development would produce developmental abnormalities similar to those seen in SIDS.

3) In the third experiment, presented in chapter 4, morphometric and stereological analyses were performed in the DMV of rats during normal postnatal development. The total volume of the DMV and the numerical density and total number of synapses were determined at various postnatal ages (postnatal days 1, 5, 10, 15, 20, 25, 30, and young adults). The purpose of this analysis was to delineate the time course of both the progressive and regressive phases of synaptogenesis during normal development. By knowing the age at which peak synaptic densities occur, one could introduce exogenous recombinant growth factors in an attempt to prevent the normal elimination of synapses during the regressive phase. If SIDS results from the failure to eliminate normally extraneous synapses from the brainstem, then preventing the regressive phase of brainstem synaptogenesis in rats might be expected to produce cardiorespiratory instability in these animals.

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Chapter 2

Morphometric Analyses of the Dorsal Motor Nucleus of the Vagus in Sudden Infant Death Syndrome

2.1. Introduction

Abnormalities in cardiorespiratory physiology have been documented for SIDS cases in prospective studies, where large numbers of randomly selected infants are monitored for a given physiological variable over time and a number of cases ultimately succumb to SIDS during the first postnatal year (for example, see Schechtman et al., 1991). Additional physiological studies have been performed with near-SIDS cases, where individual infants were resuscitated from an episode of cardiorespiratory failure and identified as being susceptible to SIDS (for example, see Guilleminault et al., 1979; Kelly and Shannon, 1979). These studies have reported altered sleep patterns (Harper et al., 1981), abnormal respiratory and cardiac functioning (Leistner et al., 1980), prolonged apnea (Guilleminault et al., 1975; Schechtman et al., 1991) and a higher frequency of apnea (Guiiliminault et al., 1979; Kelly and Shannon, 1979). Theories involving apnea suggest failure of autonomic regulation of respiration during sleep (Steinschneider, 1972), or decreased arousal from sleep during hypoxic episodes from apnea (Harper et al., 1981), from rebreathing exhaled gases (Kinney et al., 1991; Guntheroth and Splers,

1996), or from inflammation of the respiratory tract (Harper et al., 1981). These hypotheses suggest an instability in respiratory functioning in susceptible infants, most likely due to a subtle developmental disorder of unknown etiology. According to this susceptibility hypothesis, exposure to an unknown environmental trigger factor would precipitate the episode of terminal apnea. Although the trigger factor(s) have not been identified, increased risks include sleeping in the prone position (Vandenplas et al., 1996; Kemp, 1996; Guntheroth and Splers, 1996; Oyen et al., 1997; Schellscheidt et al., 1997; Sawczenko and Fleming, 1996), recent illness such as respiratory infection, nasal congestion, cough, swaddling, sleeping on a soft pillow or mattress (Gilbert et al., 1992; Ponsonby et al., 1993).

Recent hypotheses concerning the pathogenesis of SIDS have focused on abnormalities in the development of the central nervous system (for reviews see Kinney et al., 1992; Becker, 1990). Possible abnormal neuronal circuitry in the areas of brainstem controlling rhythmic breathing, cardiorespiratory coupling, and arousal, has been the focus of recent research. Reports of central nervous system abnormalities in SIDS include increased brain weight (O'Kusky and Norman, 1992; Shaw et al., 1989; Kinney et al., 1991; Aranda et al., 1990), increased volume of the pons and medulla (O'Kusky et al., 1995), and an increased rate of growth in the volume of the hypoglossal nucleus, the nucleus pontis, and the total volume of the pons (O'Kusky and Norman, 1992; O'Kusky et al., 1995). Additional studies have demonstrated increased connectivity among neurons in some brainstem nuclei in SIDS victims. For example, significant increases in the density of dendritic spines have been reported in numerous brainstem nuclei which are known to contain

neurons controlling cardiovascular activity, rhythmic breathing, and arousal. These nuclei include the magnocellular reticular nucleus (Takashima and Becker, 1985), nucleus of the tractus solitarius (Quattrochi et al, 1985; Takashima and Becker, 1985; Becker and Zhang, 1996), dorsal motor nucleus of the vagus (Takashima et al., 1994; Becker and Zhang, 1996; Takashima and Becker, 1985), nucleus ambiguus, nucleus gigantocellularis, nucleus pontis, hypoglossal nucleus, nucleus of the lateral lemniscus, and spinal trigeminal nucleus (Quattrochi et al., 1985). In addition, the studies by Quattrochi et al. (1985) and Takashima and Becker (1985) demonstrated that the density of dendritic spines decreased by 65-80% from peak levels at 36 postconceptional weeks of age in normal controls. These findings suggest that the regressive phase of synaptogenesis begins immediately before birth in these brainstem nuclei, reaching typical adult densities by approximately one year of age. The SIDS victims had the same decreasing trend in dendritic spine density, but the overall density was significantly greater than in control cases by approximately 75-180% (Quattrochi, 1985). Significant increases in the number of synapses in the hypoglossal nucleus (O'Kusky and Norman, 1995) and the density of synapses in the central reticular nucleus of the medulla (O'Kusky and Norman, 1994) correlate with the increased dendritic spine density. The findings of increased density of synapses and dendritic spines in SIDS victims most likely reflect a persistence of normally transient connections during the regressive phase of synaptogenesis.

The purpose of the present study was to compare postnatal changes in volume of the dorsal motor nucleus of the vagus (DMV) in SIDS cases and age-

matched control cases without neurological disease. Furthermore, comparisons of postnatal changes in the numerical density of neurons (N_V, cells per unit volume of tissue), the total number of neurons, and mean neuronal profile areas were performed. Data were collected separately for both motor and non-motor neurons. Given the previous studies demonstrating overgrowth of various brainstem nuclei in SIDS, one would expect to find in SIDS cases: 1) a significant increase in nuclear volume, 2) a significant decrease in the N_V of neurons, 3) no change in total neuron number, and 4) significantly larger mean neuronal profile areas.

The DMV not only functions as the main visceral and thoracic parasympathetic nucleus, but also contains neurons of the dorsal respiratory group, which are responsible for initiating and maintaining inspiration. DMV neurons also play a role in the control of cardiac physiology, maintaining the beat-to-beat regulation of heart rate. Any abnormalities in the maturation of the DMV could conceivably cause cardiorespiratory instability that characterizes susceptible infants.

2.2. Materials and Methods

2.2.1. Study population

Brains from 33 SIDS cases and 14 non-SIDS controls were selected after autopsy for inclusion in this study. Infants were classified as SIDS if death occurred suddenly and unexpectedly, and the death remained unexplained after a thorough case investigation. This investigation included performance of a complete autopsy, which consisted of radiological, histological, microbiological, and toxicological

analyses, examination of the death scene, and review of the clinical history. Petechiae were observed on the majority of infants classified as SIDS in this study. These petechiae were located on visceral pleura, epicardium, and thymus. Excluded from the study were those victims who had been mechanically ventilated, or those that displayed edema by means of gross and microscopic evaluations. Thirty-three SIDS cases were randomly chosen for inclusion in this study. In 29 of the 33 SIDS cases, resuscitation was attempted, but in none of the cases was a pulse restored by the first responder.

Controls were infants whose cause of death was clearly identifiable. Included in this study were 14 controls, where the cause of death was diagnosed as pneumonia (n=1), non-cyanotic congenital heart disease (n=10), acute infections (n=2), or myocarditis (n=1). The brain and spinal cord of these control infants were deemed normal after histological examination. In 8 of the 14 control cases, resuscitation was attempted, but in none of the cases was a pulse restored.

No brains with a postmortem interval greater than 48 hours were included in this study. Clinical variables for the two groups are presented in Table 2.1. The mean postmortem delay (± standard error of the mean) for SIDS was 22.1 ± 4.2 hours, compared to 19.9 ± 3.1 hours for control victims. The mean postmortem delay for SIDS cases differed from controls by less than 3 hours, although this difference was not statistically significant ($t= 0.421$, $df= 45$, $P> 0.05$). However, the time of death noted for SIDS is likely to be an overestimate, because it was calculated from the last time that the infant was observed to be alive by the parents. Because the majority of SIDS cases die at night during sleep, the actual time of

death would likely be later than estimated.

The mean age (± standard error of the mean) for all SIDS cases was 50.07 ± 1.40 postconceptional weeks, which did not differ significantly from the mean age for the control cases (48.25 ± 4.78 postconceptional weeks, t= 0.365, df= 45, P> 0.05). Preterm infants accounted for 13 of the 33 SIDS cases (39%) and for 5 of the 14 controls (36%). The average gestational age at birth was determined to be 35.00 ± 1.33 weeks for preterm SIDS cases and 37.40 ± 0.68 weeks for preterm controls.

2.2.2. Tissue preparation

At autopsy the brains were removed and the brainstems were cut from the attached cerebelli and cerebral hemispheres. Tissue blocks from the medulla were dissected and fixed by immersion in 2.5% gluteraldehyde in 0.1M phosphate buffer for 30 days. The medulla was cut at the midline in eight cases to allow for both light and electron microscopic analyses. Serial frozen sections (30 μm in thickness) of the complete or hemisectioned medulla were cut in the transverse plane through the entire length of the medulla. Every fourth section was then mounted on chrome alum gelatin-coated glass slides and stained for Nissl substance using 0.1% aqueous thionine in acetate buffer (pH 3.7). All histological specimens were coded to prevent bias during the morphometric analyses.

2.2.3. Morphometric analyses

Total volume of the DMV was measured in mm^3 using the serial Nissl

sections through the medulla. The caudal extent of the DMV was defined as the most caudal limit containing three or more motor neurons, which is situated in the caudal medulla, lateral to the central canal. The rostral extent of the DMV was defined as the disappearance of clustered motor neurons nearing the pontomedullary junction. Volumes were then determined using the serial Nissl sections viewed at a final magnification of x105, according to procedures previously reported (O'Kusky and Norman, 1992; O'Kusky et al., 1995; O'Kusky and Norman, 1995). The area of the DMV on every fourth section was measured in mm^2 by using a digitizing tablet with an image analysis system (Bioquant System IV, R&M Biometrics, Nashville, TN). The boundary of the DMV was determined using the criteria of Olszewski and Baxter (1954), as illustrated in Figure 2.1. The number of sections analyzed per nucleus ranged from 80-120. The total volume of the DMV was calculated by the following equation:

$$V = \sum A \times T \times P,$$

where $\sum A$ is the sum of the area measurements, T is the thickness of the section, and P is the periodicity of the sample. In this case, the thickness of each section was 30 μm, with a periodicity of 4. Total length of the DMV was calculated using the formula:

$$L = \sum N \times T \times P,$$

where $\sum N$ is the total number of sections throughout the nucleus.

The numerical density of motor and non-motor neurons were determined using the Abercrombie method (Abercrombie, 1946). Briefly, individual sections were examined at a final magnification of x2,645. Neurons were counted when their

nuclear profiles contained a distinct nucleolus. The numerical density (N_V) was calculated from the following equation:

$$N_V = N_A / (D + T)$$

where N_A is the number of neuronal profiles per unit area of DMV, D is the mean nucleolar diameter, and T is the thickness of the section. Data were collected separately for motor and non-motor neurons. These two populations could be distinguished by the quantity and distribution of Nissl substance, size and shape of the neuron, and location throughout the DMV, as illustrated in Figure 2.2. Specifically, motor neurons were identified by clumped and intensely stained Nissl substance, larger cell body profile areas, and ovoid, multipolar shape. Non-motor neurons were identified by having relatively little Nissl substance, pale staining intensity, uniformly small cell body profile areas, and a rounded shape. The total numbers of motor and non-motor neurons were calculated from estimates of neuronal N_V and total volume of the DMV.

The mean profile areas for the motor and the non-motor neurons were determined at a final magnification of x2,645. Using the Bioquant digitizing system, the boundaries of the neuron at a plane of focus that contained a clear and distinct nucleolus, was outlined and the area determined. The mean profile areas were based on 100 motor neurons and 100 non-motor neurons. These neurons were randomly chosen from sections distributed throughout the caudal-rostral extent of the DMV.

2.2.4. Statistical analysis

Postnatal changes in the various morphometric variables were compared in SIDS cases and controls using linear regression analysis. The statistical significance of direct comparisons between the means of SIDS cases and controls was determined by Student's t-test.

2.3. Results

The boundary of the DMV was distinguished using the position of motor neurons to determine the outer limits, as illustrated in Figure 2.1. For the rostral two-thirds of the DMV, the nucleus was located ventrolateral to the floor of the Ivth ventricle, dorsolateral to the nucleus intercalatus and hypoglossal nucleus, ventromedial to the nucleus of the tractus solitarius, and dorsomedial to the medullary reticular formation (Fig. 2.1). The differences in the two cell types within the boundaries of the nucleus, the motor and non-motor neurons, are illustrated in figure 2.2. The motor neurons were readily distinguished from the non-motor neurons in all cases used in this study. Overall, the motor neurons exhibited a multipolar shape with clumped and intensely stained Nissl substance. No atrophy, inflammation, gliosis, or edema was observed. This was consistent in all specimens used in the present study. Distinction between the neurons of the DMV from those of the ventromedial hypoglossal nucleus was apparent, based on a smaller size and less Nissl substance. Distinction from the dorsolateral nucleus of the tractus solitarius was based on larger motor neurons containing increased amounts of Nissl substance. The reticular formation, which does not have a distinct boundary, lies

ventral to the DMV. These neurons are dissimilar to those of the DMV due to their less intense staining and smaller neuronal size.

Mean body weights and brain weights for SIDS cases and controls are presented in Table 2.1. The mean body weight did not differ significantly between SIDS and controls ($t= 1.886$, $df= 45$, $P> 0.05$), but the mean brain weight was found to be significantly greater in SIDS cases than in controls (24%, $t= 2.222$, $df= 45$, $P< 0.05$).

Postnatal changes in the total volume of the DMV for SIDS cases and controls are illustrated in Figure 2.3. Individual data points represent the volumes of individual DMV nuclei. Postnatal development of the DMV was characterized by an approximately linear increase in volume from birth to one year of age for both SIDS cases ($Y= 2.69 + 0.03X$, $r= 0.278$, $P< 0.05$) and controls ($Y= 2.27 + 0.02X$, $r= 0.515$, $P< 0.05$). Linear regression analysis revealed no significant difference between the slopes of the lines for SIDS cases and controls ($t= 0.813$, $df= 76$, $P> 0.05$), indicating that the rate of growth was the same for the DMV in both groups. By direct comparison, there was a substantial increase in the volume of the DMV in SIDS cases when compared to controls (33%, $t= 5.510$, $df= 78$, $P< 0.0001$). After examining Figure 2.3, it was noted that, although the mean ages for SIDS and control groups did not differ significantly, the two groups were not exactly age-matched. For example, there were no control cases between 56 and 80 weeks of age, while there were no SIDS cases older than 72 weeks of age. The two groups were very closely age-matched for cases less than 56 postconceptional weeks of age. Direct comparison of DMV volume in cases less than 56 weeks of age

revealed a similar increase in the total volume of the nucleus in SIDS cases, when compared to controls (40%, t= 7.459, df= 61, P< 0.0001). Despite the significant increase in mean DMV volume in SIDS cases, it is interesting to note that approximately 23% of the nuclei from SIDS cases fall within or lie below the 99% confidence interval of control values.

Length of the DMV nucleus (Fig. 2.4) in SIDS did not differ from controls (t= 0.367 , df= 78, P> 0.05), but the mean profile area of the DMV per section (Fig. 2.5) was found to be 27% larger in SIDS cases than in controls (t= 4.204, df= 78, P< 0.001). When examining individual sections from SIDS cases and age-matched controls at a corresponding level through the DMV (Fig. 2.6), this increase in the surface area was obvious. However, given the variability in the area of the DMV on individual sections within a given case and on sections between cases of different ages and from the two groups, morphometric analysis were necessary to confirm this finding. The increased volume of the DMV in SIDS would appear to be due to an increased girth, rather than to an increased length along the caudal-to-rostral axis.

Postnatal changes in the N_V for all DMV neurons are illustrated in Figure 2.7. Normal development was characterized by a decrease in the N_V of neurons from birth to approximately one year of age. The N_V was found to decrease from approximately 15,000 neurons per mm^3 at birth to 9,000 neurons per mm^3 at one year of age. However, linear regression analysis showed that this decrease was not significantly linear for controls (Y= 21.67 – 0.09X, r= 0.390, df= 12, P> 0.05). Similarly, the apparent decrease in the N_V of total neurons in SIDS cases was not

significantly linear (Y= 15.72 – 0.05X, r= 0.186, df= 31, P> 0.05). However, by direct comparison the N_V of total neurons was found to be significantly less in SIDS cases than in control cases (33%, t= 4.730, df= 78, P< 0.0001). For cases less than 56 weeks of age, direct comparison revealed a significant decrease in the N_V of total neurons in SIDS cases (35 %, t= 3.801, df= 36, P< 0.0001).

Postnatal changes in the N_V for motor neurons in the DMV are illustrated in Figure 2.8. N_V of motor neurons in control cases was found to decrease from approximately 12,000 neurons per mm^3 at birth to 9,000 neurons per mm^3 at one year of age. This apparent decrease, however, was not significantly linear by linear regression analysis (Y= 30.85 + 0.02X, r= 0.002, df= 12, P> 0.05). Similarly, the apparent decrease in the N_V of motor neurons in SIDS cases was not significantly linear (Y= 8.82 – 0.03X, r= 0.040, df= 31, P> 0.05). Direct comparison of the motor neuron N_V, however, showed a significant decrease in SIDS cases when compared to control cases (44%, t= 6.885, df= 44, P< 0.001). For cases less than 56 postconceptional weeks of age, direct comparison revealed a significant decrease (35%, t= 3.801, df= 36, P< 0.001) in SIDS.

Postnatal changes in the N_V for non-motor neurons in the DMV are illustrated in Figure 2.9. Normal development was characterized by a decrease in the N_V of non-motor neurons from approximately 8,000 neurons per mm^3 at birth to 6,000 neurons per mm^3 at one year of age. This apparent decrease, however, was not significantly linear by linear regression analysis (Y= 8.55 – 0.04X, r= 0.073, df= 12, P> 0.05). As well, the apparent decrease in the N_V of non-motor neurons in SIDS cases was not significantly linear (Y= 6.91 – 0.02X, r= 0.016, df= 31, P> 0.05).

However, direct comparison of the N_V for non-motor neurons revealed a significant decrease in SIDS cases when compared to control cases (19%, t= 1.761, df= 44, P< 0.05). For cases less than 56 postconceptional weeks of age, however, direct comparison revealed no significant decrease (20%, t= 1.490, df= 36, P> 0.05).

Postnatal changes in the total number (TN) of all neurons in the DMV are illustrated in Figure 2.10. Normal development was characterized by no obvious change in neuronal number, remaining at approximately 50,000 neurons from birth to one year of age. Linear regression, however, revealed that this trend is not significantly linear for control cases (Y= 51.56 – 0.01X, r= 0.012, df= 12, P> 0.05). Similarly, the TN of neurons in SIDS cases was not significantly linear (Y= 39.44 + 0.26X, r= 0.150, df= 31, P> 0.05). Direct comparison of the TN showed no significant difference between SIDS cases and control cases (t= 0.151, df= 44, P> 0.05). Therefore, SIDS cases contain a normal complement of DMV neurons in a larger volume of tissue.

Postnatal changes in the TN of motor and non-motor neurons in the DMV are illustrated in Figure 2.11 and 2.12, respectively. As with the TN of all DMV neurons, normal development was characterized by no obvious change in neuronal number from birth to one year of age. These values remained at approximately 30,000 for motor neurons, and 20,000 for non-motor neurons. Linear regression analysis revealed that the trend for motor neurons was not significantly linear in control cases (Y= 30.85 + 0.02X, r= 0.002, df= 12, P> 0.05), or in SIDS cases (Y= 21.05 – 0.17X, r= 0.037, df= 31, P> 0.05). Similarly, the values of TN for non-motor neurons was not significantly linear in control cases (Y= 20.71 – 0.01X, r= 0.001,

df= 12, P> 0.05), or in SIDS cases (Y= 18.37 – 0.09X, r= 0.008, df= 31, P> 0.05). Direct comparison of TN for both motor neurons and non-motor neurons showed no significant change in SIDS cases compared to controls (t= 0.824, df= 44, P> 0.05) and (t= 0.944, df= 44, P> 0.05), respectively.

Mean profile area of motor neurons in the DMV was determined to be significantly larger in SIDS than in controls (31%, t= 7.331, P< 0.0001). As shown in Figure 2.13, there was relatively little overlap between the mean profile areas for the two groups. During normal postnatal development in controls, there was a 30% increase in motor neuron size from birth to one year of age. This increase was not evident in SIDS cases. Although regression analysis showed that the increase in cell size in control victims was linear (Y = 236.55 + 1.44X, r= 0.648 df = 12, P< 0.05), the trend was not significantly linear for SIDS (Y = 458.96 – 1.16X, df = 30, r = 0.233) due to the substantial variability of mean profile areas for individual cases. The mean profile areas of the non-motor neurons as a function of age are illustrated in Figure 2.14. There was a significant increase in mean profile area for SIDS cases, when compared to controls (30%, t= 4.120, df= 44, P< 0.001).

The mean diameter of the nucleolus in motor neurons (Fig. 2.15) was determined to be significantly larger in SIDS cases, when compared to controls (15%, t= 5.675, df= 44, P< 0.0001). There was no significant difference found in the mean nucleolar diameter for non-motor neurons.

While measuring the diameter of the nucleoli in the DMV neurons of SIDS cases and controls, an interesting observation was made. Although not quantitatively analyzed, it appeared as though there was an increased frequency

and size of intranuclear inclusion bodies adjacent to the nucleolus in SIDS cases (Fig. 2.16). These consisted of small spherical bodies, approximately 0.3 to 0.8 μm in diameter, with a texture and staining intensity similar to material within the nucleolus. These inclusions were observed more frequently in motor neurons than in non-motor neurons. Although similar granular bodies were occasionally observed in control neurons, the size and frequency of these inclusions appeared to be greater in SIDS cases than in controls. Since the size of these paranucleolar inclusions was near the limits of resolution for the light microscope, further analyses by electron microscopy remain to be performed.

2.4. Discussion

SIDS cases analyzed in this study displayed an increased volume of the dorsal motor nucleus of the vagus when compared to control infants (33%). This finding is consistent with previous reports from our lab showing increased volume of the medulla, pons, and the nucleus pontis (O'Kusky et al., 1995), and an increased volume of the hypoglossal nucleus (O'Kusky and Norman, 1992). This is in agreement with the many reports of increased brain weight (O'Kusky and Norman, 1992; Shaw et al, 1989; Kinney et al., 1991; Aranda et al, 1990). Previous studies have shown that there are greater numbers of dendritic spines in the reticular nucleus of the medulla and non reticular areas of the brainstem (Quattrochi et al., 1985; Takashima and Becker, 1985) the nucleus of the tractus solitarius, the dorsal vagal nuclei (Takashima and Becker, 1985;Takashima et al., 1994) and the ventrolateral medulla (Takashima et al., 1994). Our lab has also established an

increased density of synapses in the reticular nucleus of the medulla, which included neurons of the ventral respiratory group, and an increased total number of synapses in the hypoglossal nucleus. This increase in synapse number was suggested to reflect an increased complexity of dendritic arborizations combined with an increased complexity of afferent axonal projections in these regions. The results of enlarged DMV volume in the present study and previous studies are consistent with increased synapse number and spine density, indicating a notable overgrowth in the brainstem of infants that ultimately succumb to SIDS.

Not all studies have documented overgrowth in the brainstem of SIDS cases. Results in direct contrast with the present study regarding DMV volume include the study by Konrat et al. (1992), who reported a decreased volume in the DMV of SIDS cases. The methods used in their study were not disclosed in any detail so as to compare the differences in the results, but it should be noted that the number of subjects investigated were substantially fewer than in the present study (6 SIDS compared to 4 controls). Lamont et al. (1995) found no significant difference in the volume of the DMV in SIDS. Again, this study used only 11 SIDS cases and 11 control cases in their evaluation. In other nuclei, Lamont et al. (1995) reported no significant differences in the volume of the hypoglossal nucleus between SIDS cases and controls, while Konrat et al (1992) reported a decrease in volume of the hypoglossal nucleus in four SIDS cases. Due to the relatively high variability in morphometric variables recorded from SIDS cases and because the diagnosis of SIDS is likely to include heterogeneous subsets, it is desirable to have a large number of cases in morphometric studies of human autopsy material. A more

respectable sample size (e.g. the 80 nuclei evaluated in the present study) will generally ensure the validity of the findings.

As shown in figure 2.3, there appear to be several SIDS cases where the volumes lie within the 99% confidence interval of control values. This is a frequent feature of SIDS research. SIDS is a diagnosis by exclusion. It is possible that sudden death due to cardiorespiratory failure could have more than one developmental etiology, defining subsets in the pathogenesis of SIDS. The 23% of SIDS cases in the present study, whose DMV volumes were indistinguishable from normal controls, probably represent such a subset. Despite this heterogeneity, it is gratifying to see that 77% of the SIDS cases in this study validated the working hypothesis of an overgrowth phenomenon.

After counting all DMV neurons in a random sample of sections, it was revealed that there was a significant decrease of 33% in the number of neurons per cubic mm of tissue with relatively little overlap between SIDS cases and controls. This decrease in N_V was observed for both the motor neurons (44%) and non-motor neurons (19%). After calculating the total number of neurons from the numerical density and the volume, it was found that there was no difference between SIDS cases and controls. The significant decrease in the N_V of neurons in SIDS resulted from a normal complement of neurons being contained in a larger volume. The greater spacing between neuronal cell bodies in a given volume of tissue indicates there is an increase in the volume of neuropil. The significant increase in the mean neuronal profile area in SIDS (31%) also contributes to some extent in this increase in volume. After calculating the size increase as a function of mean area per section

increase it was found the increase in neuronal cell size does not fully account for the total increase in the volume of the nucleus. In SIDS it was found that the ratio of area taken up by neurons to the area of neuropil was 1: 9.6. In controls, this ratio was found to be 1: 8.5. This shows that there is a larger amount of neuropil relative to neuronal area in SIDS cases than in control cases. Together, the increased neuronal profile area and the increased amount of neuropil contribute to the larger volume.

During normal neuronal development, neuronal profile areas increase. Findings in the present study were in agreement with this fact (slope = 1.4392)(Fig. 2.6 and Fig 2.7). Control motor neuronal cell size increased from approximately 250μm^2 at birth, to 375μm^2 near 1 year of age. This normal control data is inconsistent with the morphometric investigation by Konrat et al. (1992). This group found the average motor neuron profile area for controls to be 463 ± 10 μm^2. The motor neuronal profile area in the SIDS cases however, was consistent with the findings of the present study. Konrat et al. found the average profile area to be 351 ± 15μm^2, which is comparable to the mean profile area of 401 ± 7μm^2 found in the present study. As mentioned above, the mean profile area of motor neurons in SIDS was found to be significantly increased with very little overlap between SIDS and controls. It would appear that motor neurons in the DMV of SIDS cases are already significantly larger at birth. This implies that an increased expression of a neuronal growth factor or other causal factor(s) occurs relatively earlier during prenatal development.

It has been previously hypothesized that SIDS could be due to a delay in

maturation (Takashima and Becker, 1985; Takashima and Becker 1986; Quattrochi et al., 1985; Becker et al., 1993; Takashima et al., 1990; reviewed by Becker, 1990). The increased volume seen in the DMV of SIDS could be due to the delay or absence of the normal decrease in the complexity of dendritic arborizations and in the number of synapses, as was speculated to be the cause in the hypoglossal (O'Kusky and Norman, 1992). The cell size would be expected to be larger to support this increased dendritic arborization.

There is another possibility as to the cause of the increased cell size seen in SIDS. It was found that there was also an increased diameter of the nucleolus (15%) with very little overlap in values. An increase in the nucleolus size is seen during cell proliferation. The nucleolus is a specialized structure that contributes to the formation, stabilization, and packing of essential gene products. Specifically, it is the site of transcription of ribosomal DNA into ribosomal RNA, the synthesis and processing of preribosomal RNA, and the transport of ribonucleoproteins to the cytoplasm (Goessens, 1984). The outer layer, the fibrillar component layer, is size dependent on the amount of proteins associated with these functions (Goessens, 1984). Because neurogenesis is complete before birth, the variation in nucleolar size in SIDS may be indicating an increase in activity levels of the cell or an increase in production of gene products.

Another interesting observation made while measuring the diameter of the nucleolus was the presence of dense nucleolar bodies situated around the periphery of the nucleolus. These nucleolar bodies were present in the control cases, but the size, staining intensity, and frequency of occurrence appeared

greater in SIDS cases. This observation is consistent with a previous report indicating the prevalence of these nucleolar bodies in brainstem nuclei of SIDS (O'Kusky et al, 1995). Furthermore, these inclusions were described by electron microscopy in the hypoglossal nucleus of these same SIDS cases (O'Kusky et al, 1995). They were identified as large paranucleolar coiled bodies, composed of electron-dense coils separated by electron-lucent interstitial spaces. Coiled bodies appear as 0.3-0.8 μm aggregates of electron-opaque coiled fibrils that are similar in shape to cords of the nucleolar pars fibrosa (for reviews see Brasch and Ochs, 1992; Bouteille et al., 1974, 1982). Similar intranuclear inclusion bodies have been shown to be produced by nucleolar budding, beginning as coiled bodies, and then differentiating into simple and complex nuclear bodies (Vagner-Capodano et al, 1980). Simple nuclear bodies appear as finely fibrillar spherical bodies with a diameter of 0.3-0.5 μm and are considered to be normal nuclear organelles. Complex nuclear bodies appear as a peripheral proteinaceous capsule containing a granular central core with diameters as large as 1-2 μm, Nuclear bodies have been shown to be composed of protein and RNA, and are believed to function in the transport of nucleolar RNA into the cytoplasm (Brasch and Ochs, 1992; Bouteille et al., 1974, 1982). In the DMV neurons of SIDS cases an increased nucleolar RNA transport to the cytoplasm would be consistent with increased protein synthesis and hypertrophy.

Because these nucleolar bodies are known to be more numerous in hyperactive cells (Brasch and Ochs, 1992), they correlate well with the finding of increased diameter of the nucleolus. Tumor cells, for example, have an over-

abundance of these structures, as well as cells responsive to hormonal stimulation (Padykula and Clark, 1981; Cidadao and David-Ferreira, 1984; Fitzgerald and Padykula, 1983). Therefore, it is conceivable that there is an increased electrophysiological activity of neurons in the DMV of SIDS, which correlates with increased protein synthesis and metabolic activity in these cells. Studies of electrophysiological activity of neurons in the DMV remain to be performed, following development of a suitable animal model.

Collectively, the results of the present study indicate that the DMV in SIDS is characterized by a developmental disorder involving hypertrophy and overgrowth, presumably in response to the increased expression of a neuronal growth factor during late prenatal and/or early postnatal development. The presumptive pathophysiology of the DMV, which likely accompanies such a developmental disorder, could contribute to the observed respiratory and cardiac instability seen prior to death in SIDS cases. In this case the abnormal expression of an unknown neuronal growth factor would be seen as the primary causal factor in the pathogenesis of SIDS. However, some studies indicate that subtle brainstem pathology in SIDS results from bouts of hypoxia-ischemia prior to death (Obonai et al, 1997; White and Lawson, 1997; Bruce and Becker, 1992; Butterworth and Tennant, 1989). The expression of some neuronal growth factors, such as IGF-I, is known to increase in the brain following severe hypoxic-ischemic injury (Beilhartz et al., 1995; Klempt et al., 1992). If brainstem overgrowth results from increased expression of a neuronal growth factor, which itself results from hypoxia in SIDS cases prior to death, then this brainstem overgrowth would be seen as an effect

rather than a cause of SIDS. The easiest way to elucidate this cause-and-effect relationship would be to develop an animal model of SIDS, involving the experimental application of exogenous growth factor to the developing brainstem. One could conceivably produce the cardiorespiratory instability and brainstem abnormalities characteristic of SIDS.

Table 2.1. Clinical variables for SIDS cases and controls included in this study.

	SIDS	Control
Cases (n)	33	14
Age (postconceptional weeks)	50.07 ± 1.40	48.25 ± 4.78
Postmortem Interval	22.1 ± 4.2	19.9 ± 3.1
Male/Female	15/18	7/7
Full Term/Preterm	20/13	9/5
Gestational age for Preterms (weeks)	35.0 ± 1.33	37.4 ± 0.68
Body Weight (kg)	5.436 ± 0.229	4.414 ± 0.491
Brain Weight (g)	640.6 ± 23.24	516.5 ± 50.8*

*$P < 0.05$, using Student's t-test.

Figure 2.1. Dorsal motor nucleus of the vagus outlined in a SIDS victim at 40.1 postconceptional weeks of age. Nissl stain (thionin). Bar=15μm.

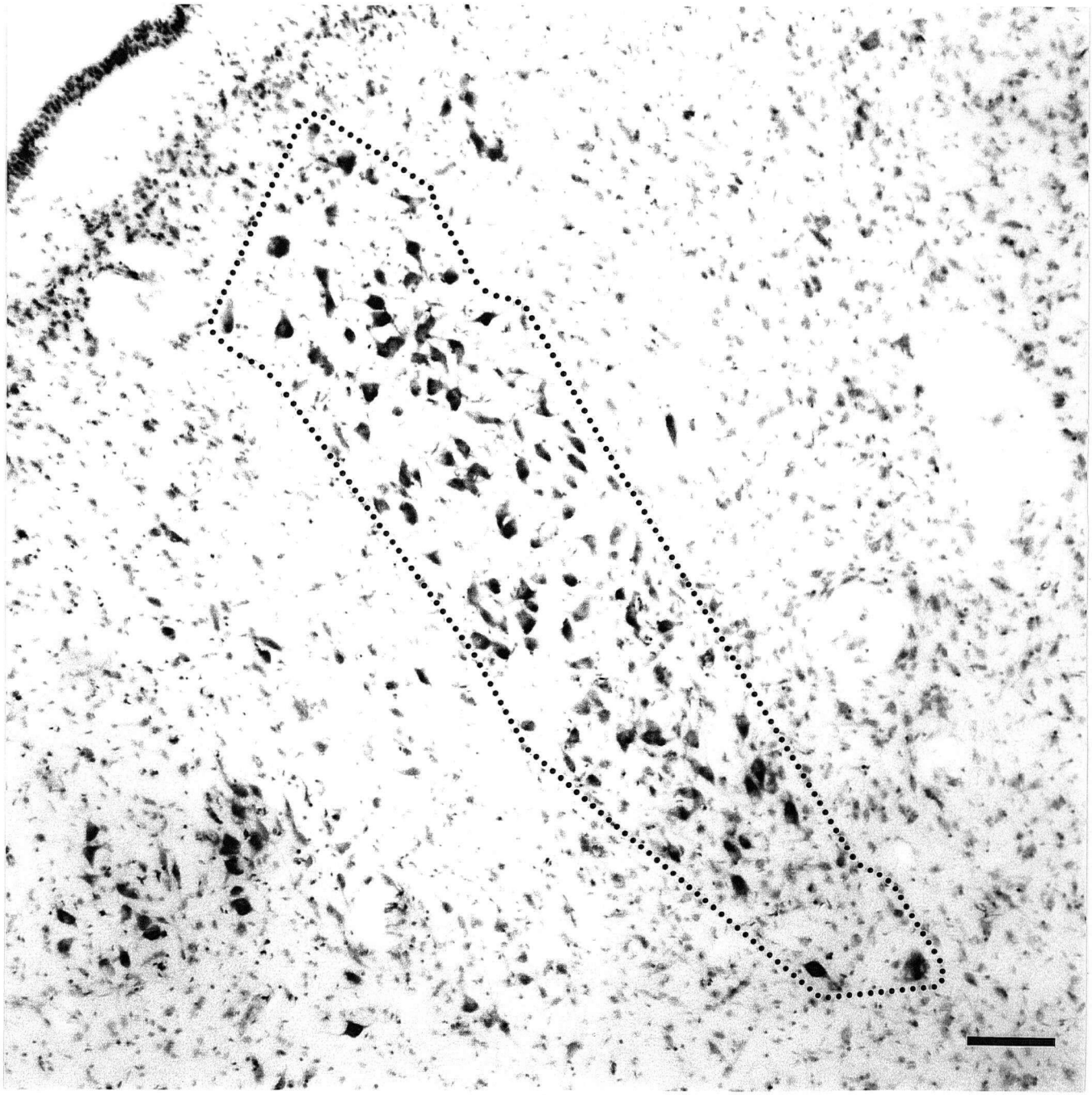
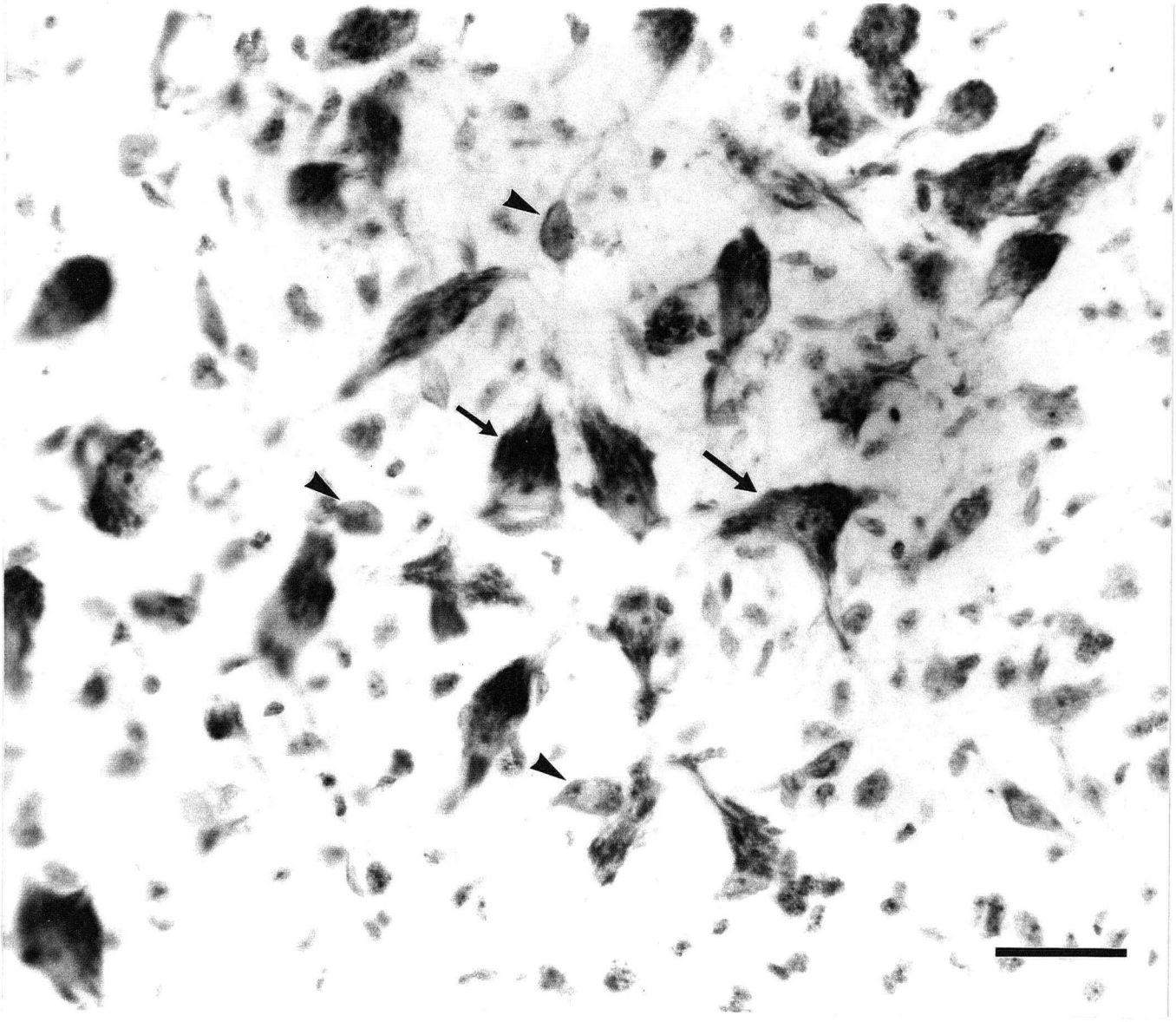


Figure 2.2. Neurons of the dorsal motor nucleus of the vagus in a SIDS victim at 40.1 postconceptional weeks of age. Motor neurons (arrows) were easily distinguished from non-motor neurons (arrow heads) by their large diameters and abundance of Nissl substance in the cytoplasm. Nissl stain (thionin). Bar=5μm.



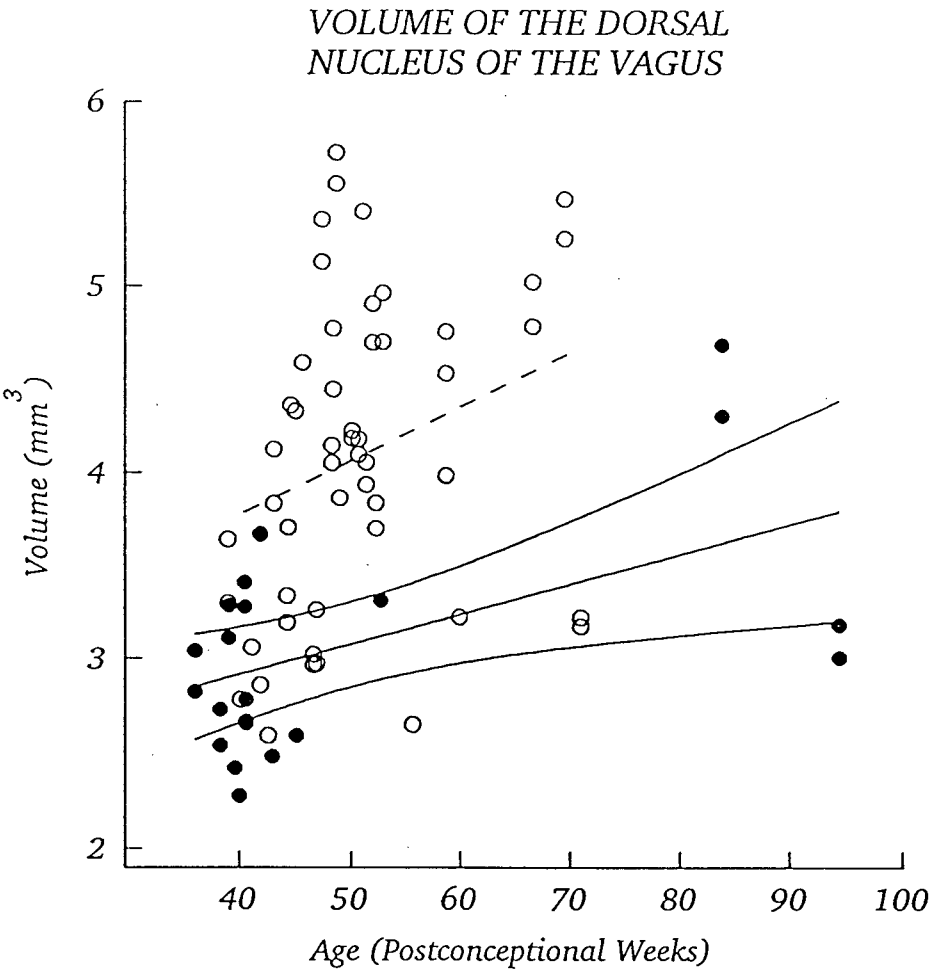


Figure 2.4. Postnatal changes in the total volume of the DMV in SIDS cases (open circles) and for control cases (filled circles) as a function of age in postconceptional weeks. Regression lines are indicated for SIDS data (dashed line) and for control data (solid line ± 99% confidence interval).

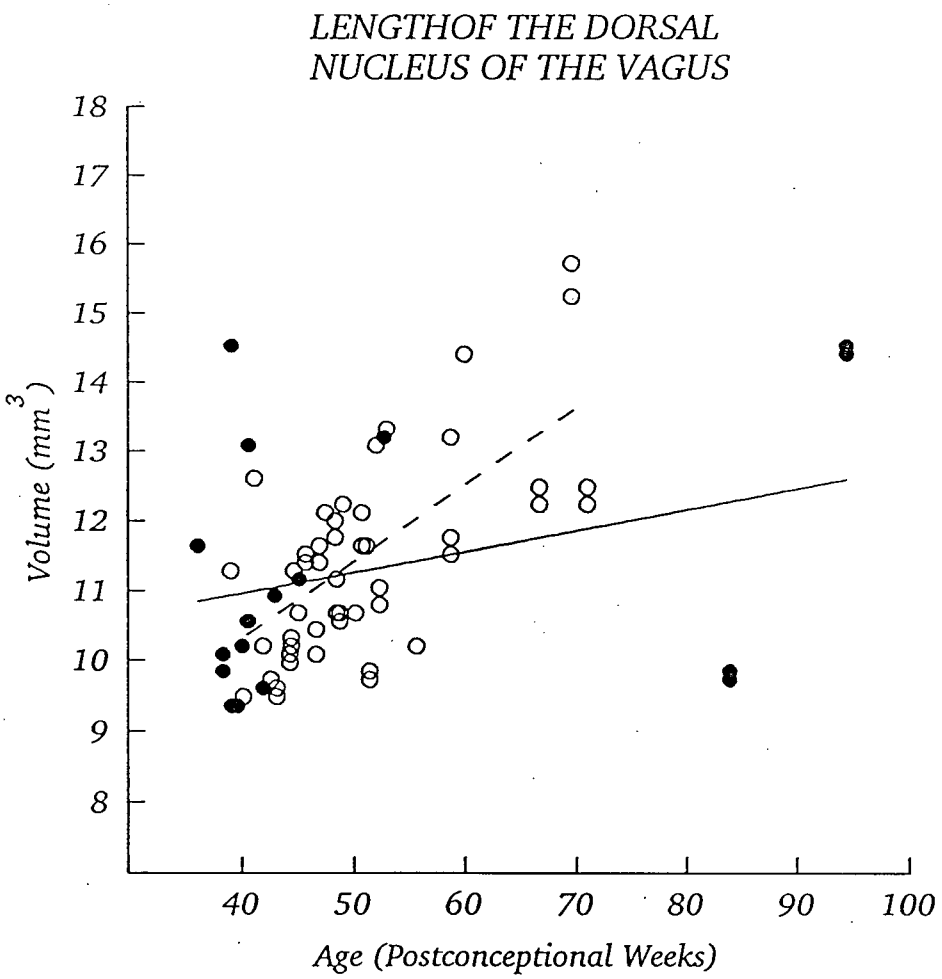


Figure 2.4. Length of the DMV for SIDS cases (open circles) and for control cases (filled circles) as a function of age in postconceptional weeks. Regression lines are indicated for SIDS data (dashed line) and for control data (solid line).

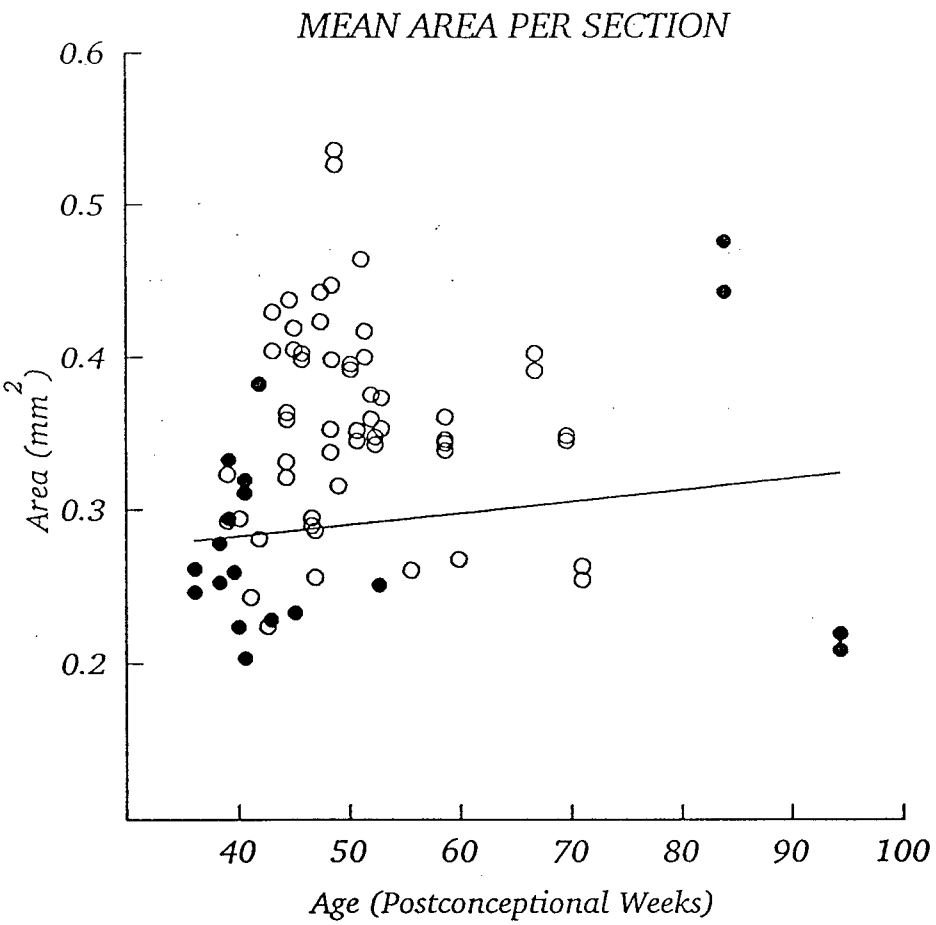
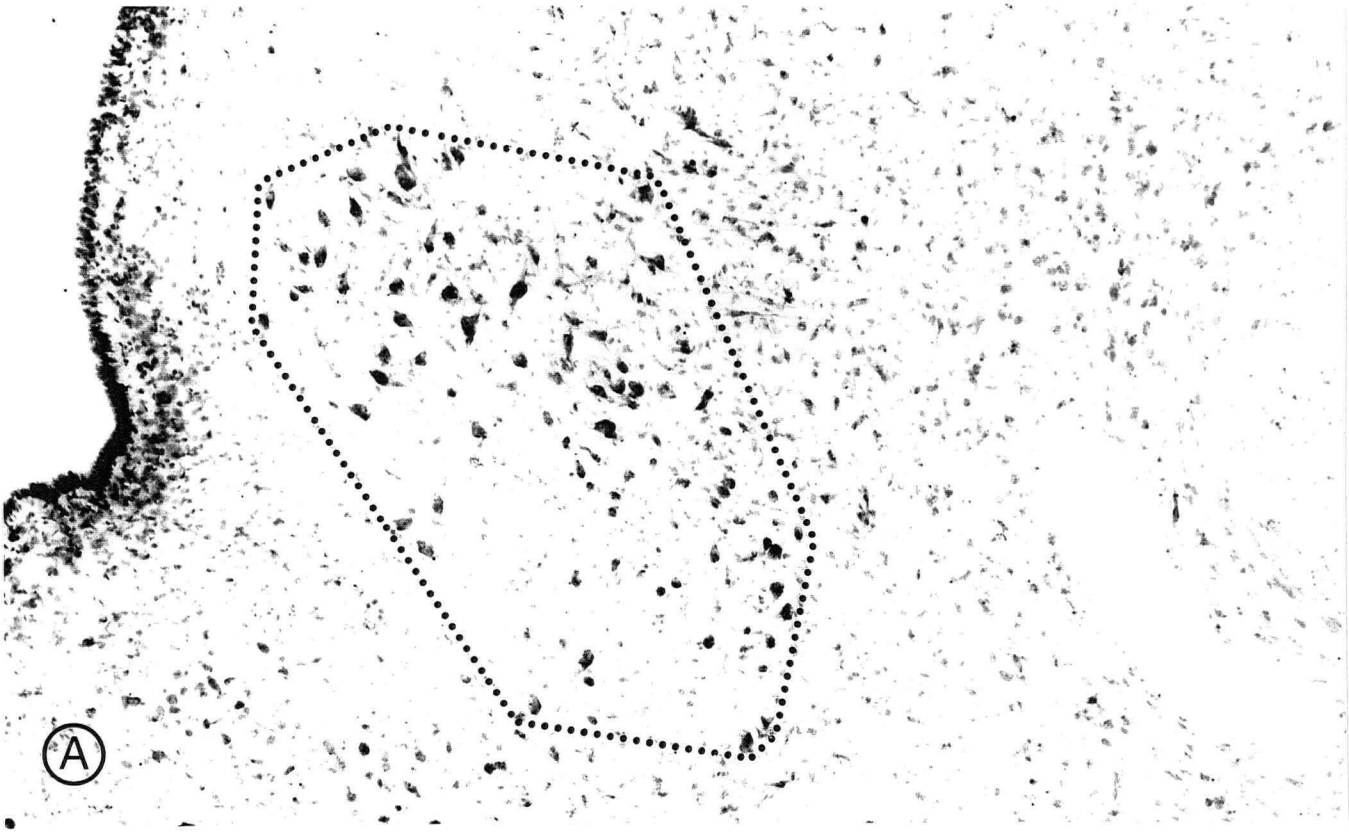


Figure 2.5. Mean area per section of the DMV for SIDS cases (open circles) and for control cases (filled circles) as a function of age in postconceptional weeks. Regression line is indicated for control data.

Figure 2.6. Dorsal motor nucleus of the vagus outlined in a control case at 52.7 postconceptional weeks of age (A) and a SIDS case at 51.4 postconceptional weeks of age (B). Cases were chosen because the calculated total volume of the DMV was close to the mean volume of the corresponding group. Note the increased nuclear area of the DMV (outlined) in the SIDS case when compared to the control case. On this figure, areal measurements revealed a 35% increase in area of the DMV for the SIDS case when compared to the control case. Nissl stain (thionin). Bar=10μm.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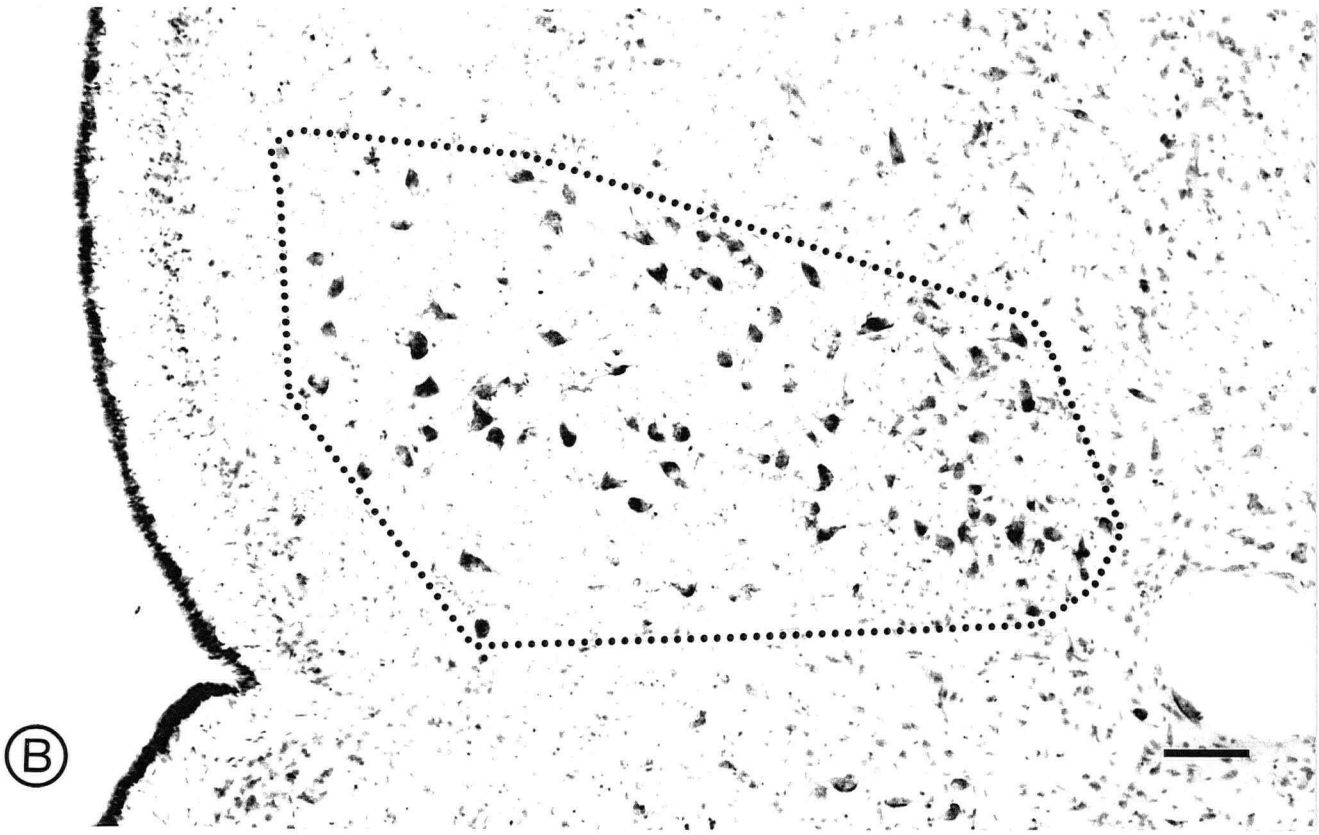
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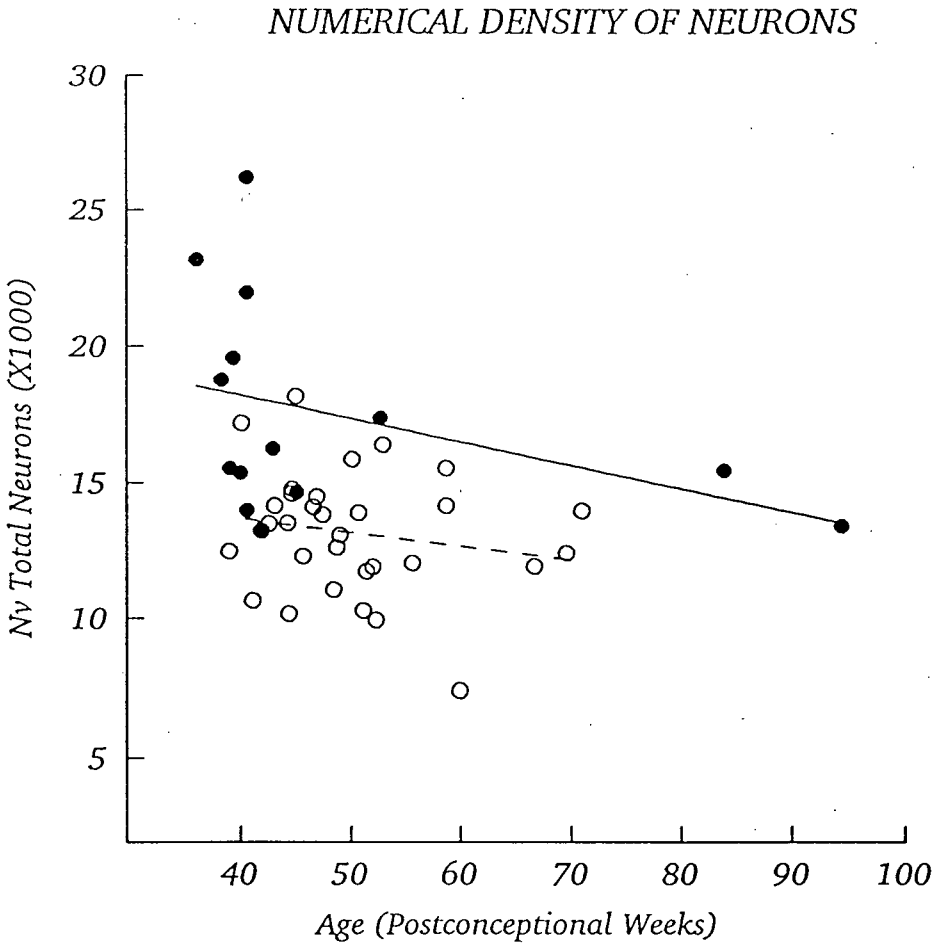


Figure 2.7. Numerical density (N_V) of motor and non-motor neurons in the DMV for SIDS cases (open circles) and for control cases (filled circles) as a function of age in postconceptional weeks. Regression lines are indicated for SIDS data (dashed line) and for control data (solid line).

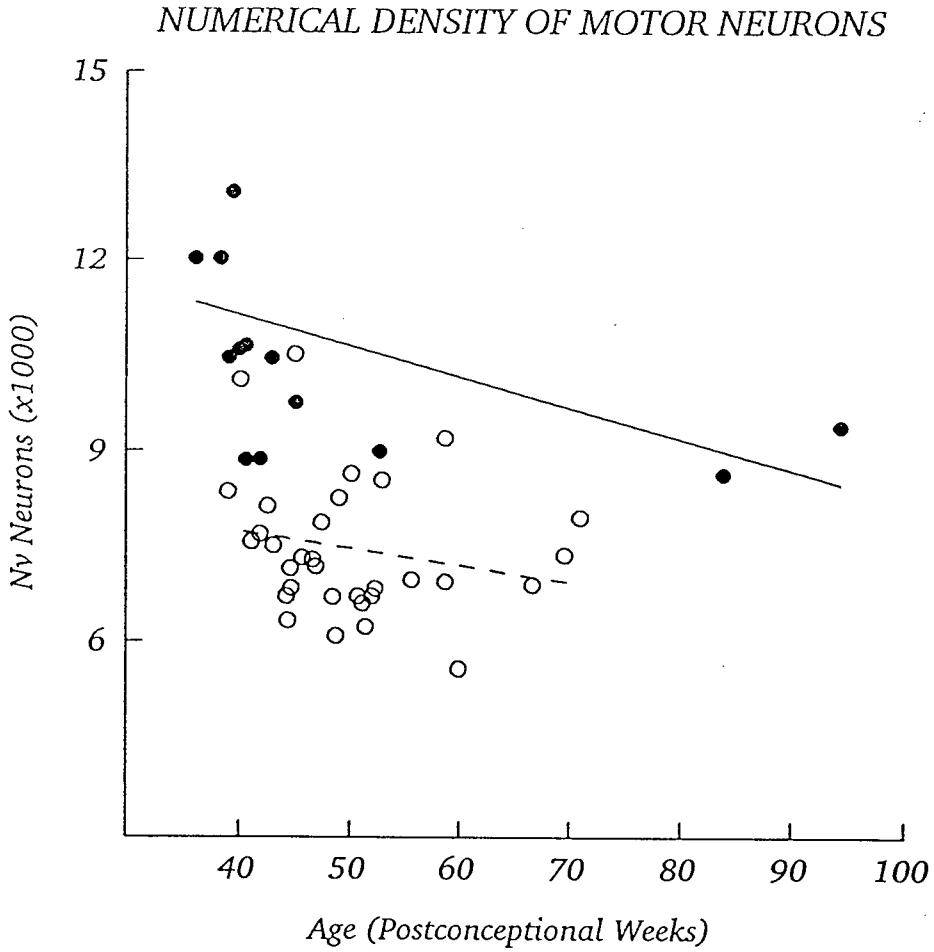


Figure 2.8. Numerical density (N_V) of motor neurons in the DMV for SIDS cases (open circles) and for control cases (filled circles) as a function of age in postconceptional weeks. Regression lines are indicated for SIDS data (dashed line) and for control data (solid line).

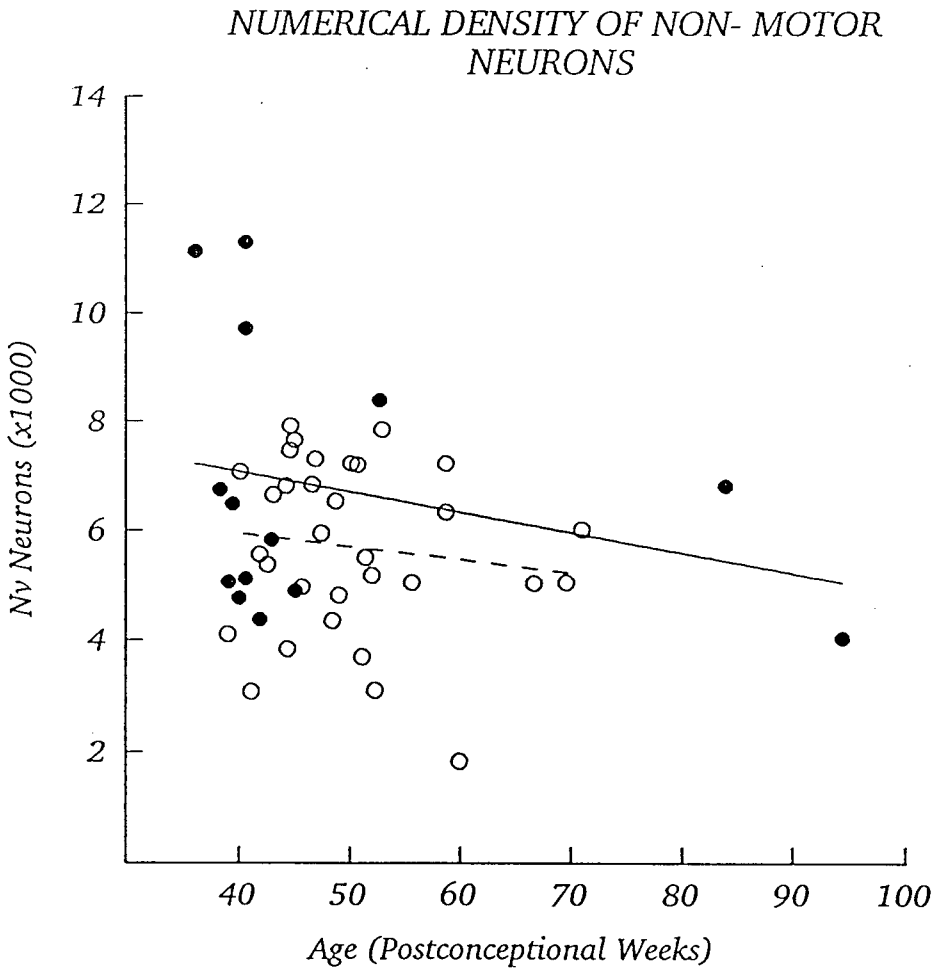


Figure 2.9. Numerical density (N_V) of non-motor neurons in the DMV for SIDS cases (open circles) and for control cases (filled circles) as a function of age in postconceptional weeks. Regression lines are indicated for SIDS data (dashed line) and for control data (solid line).

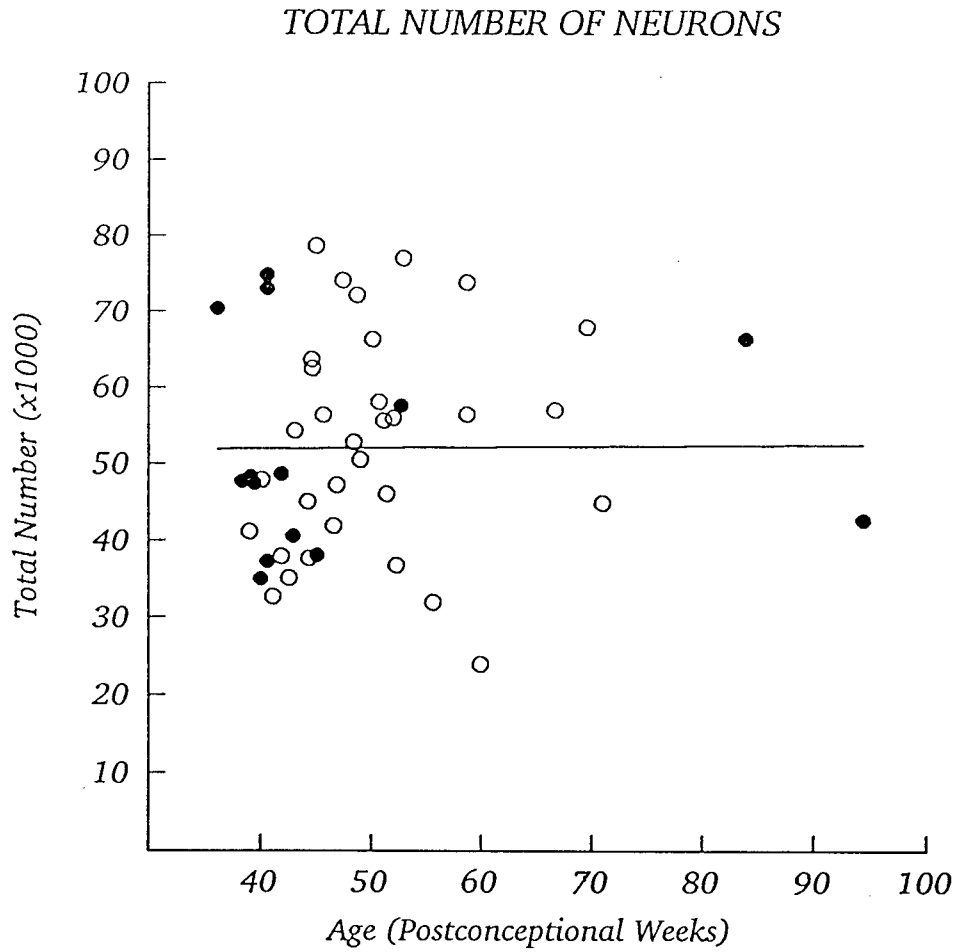


Figure 2.10. Total number (TN) of motor and non-motor neurons in the DMV for SIDS cases (open circles) and for control cases (filled circles) as a function of age in postconceptional weeks. Regression line is indicated for control data (solid line).

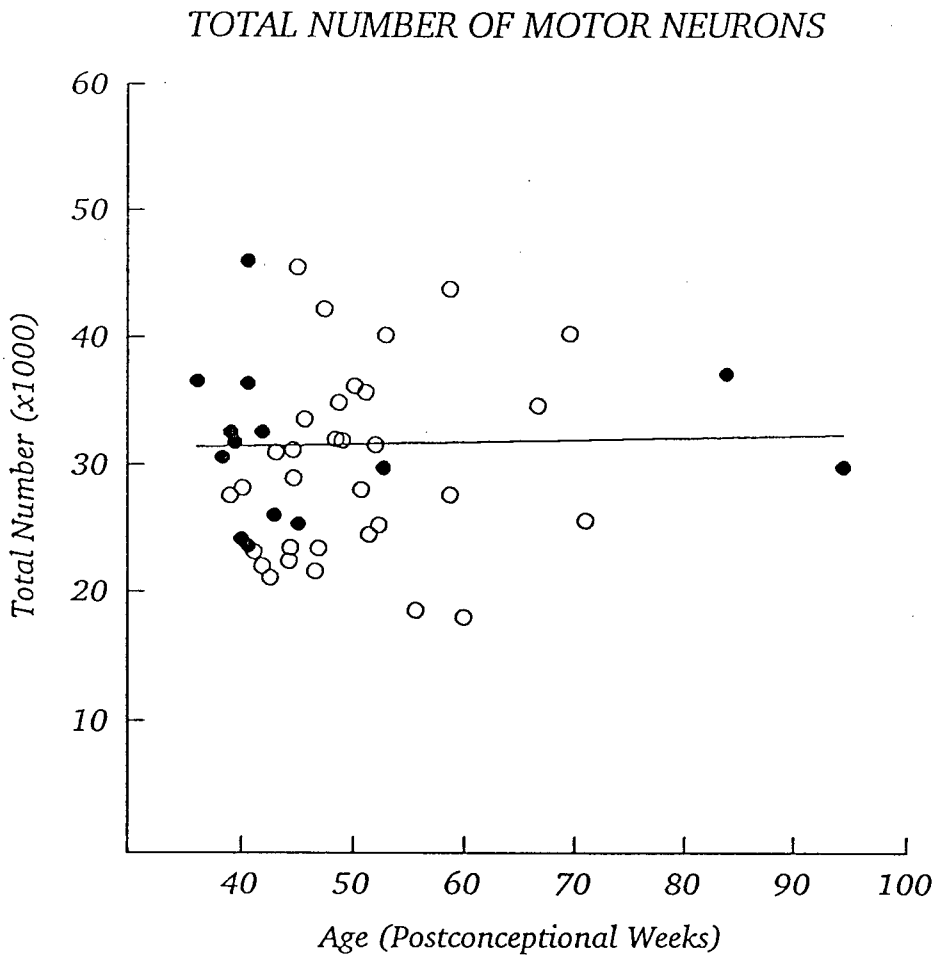


Figure 2.11. Total number (TN) of motor neurons in the DMV for SIDS cases (open circles) and for control cases (filled circles) as a function of age in postconceptional weeks. Regression line is indicated for control data (solid line).

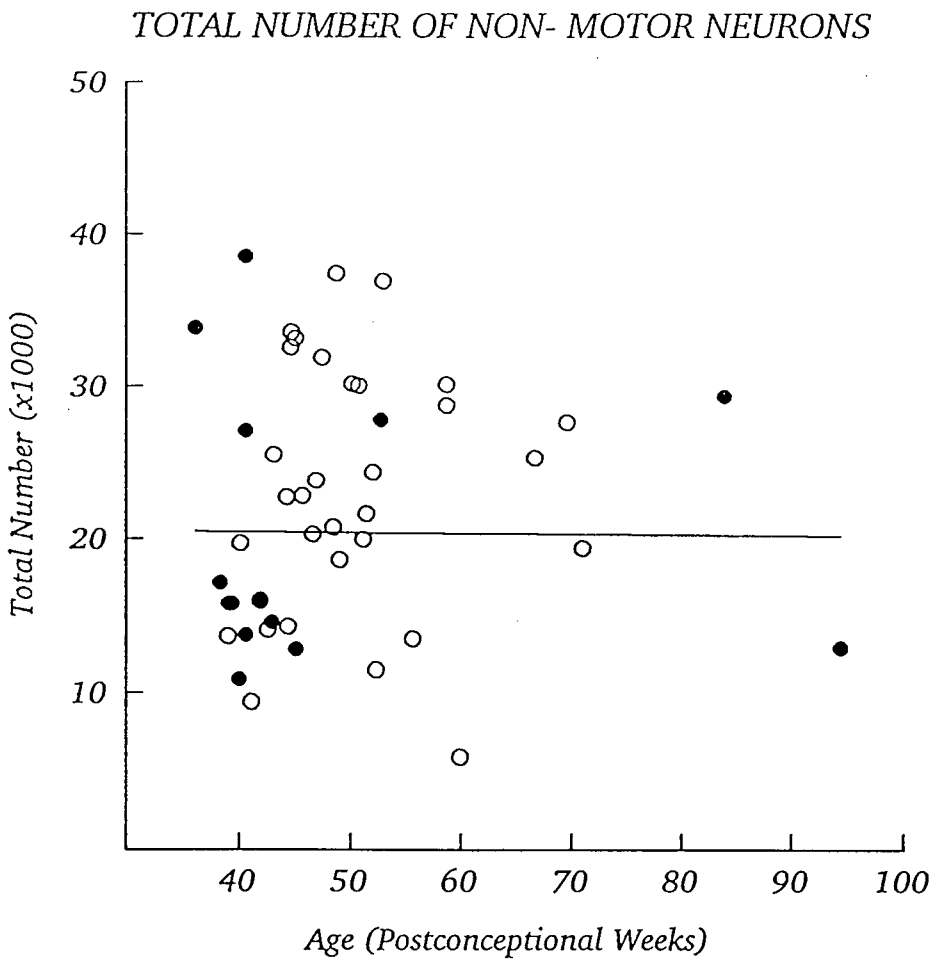


Figure 2.12. Total number (TN) of non-motor neurons in the DMV for SIDS cases (open circles) and for control cases (filled circles) as a function of age in postconceptional weeks. Regression line is indicated for control data (solid line).

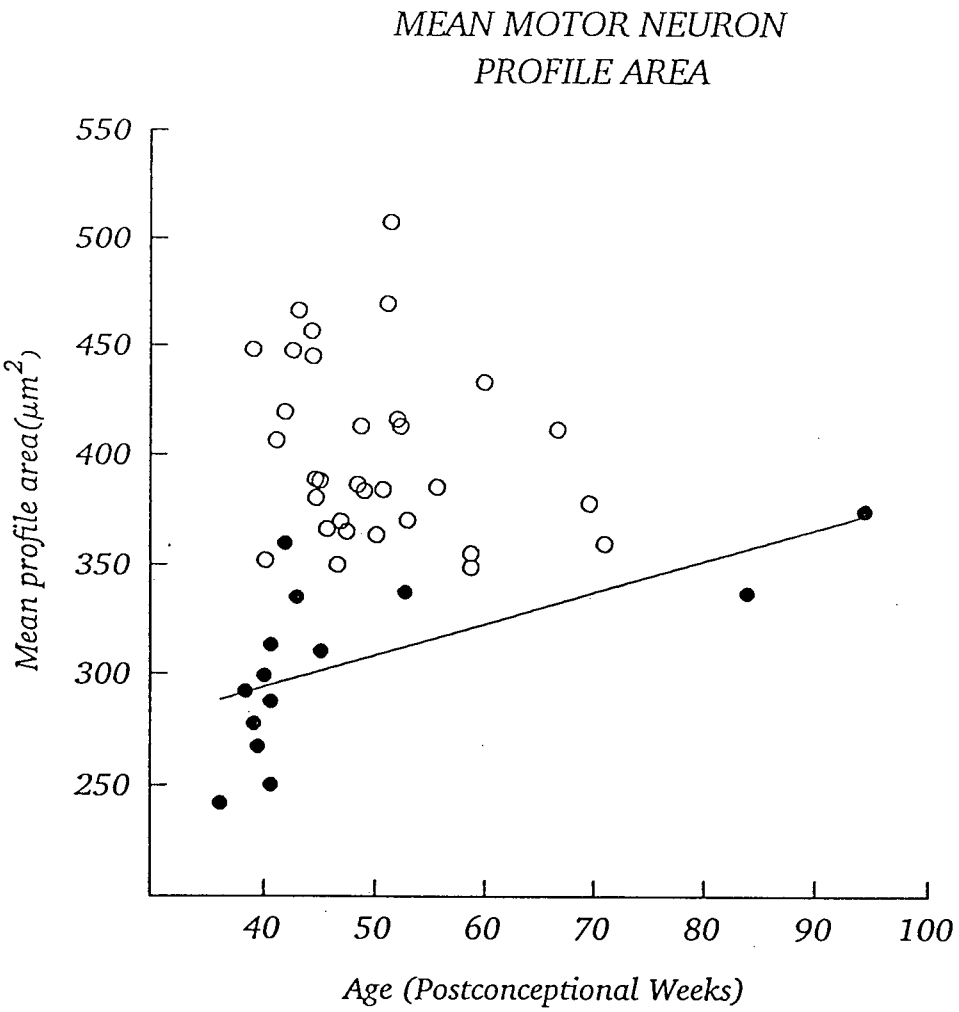


Figure 2.13. Mean profile area for cell bodies of motor neurons in the DMV of SIDS cases (open circles) and of control cases (filled circles) as a function of age in postconceptional weeks. Regression line is indicated for control data (solid line).

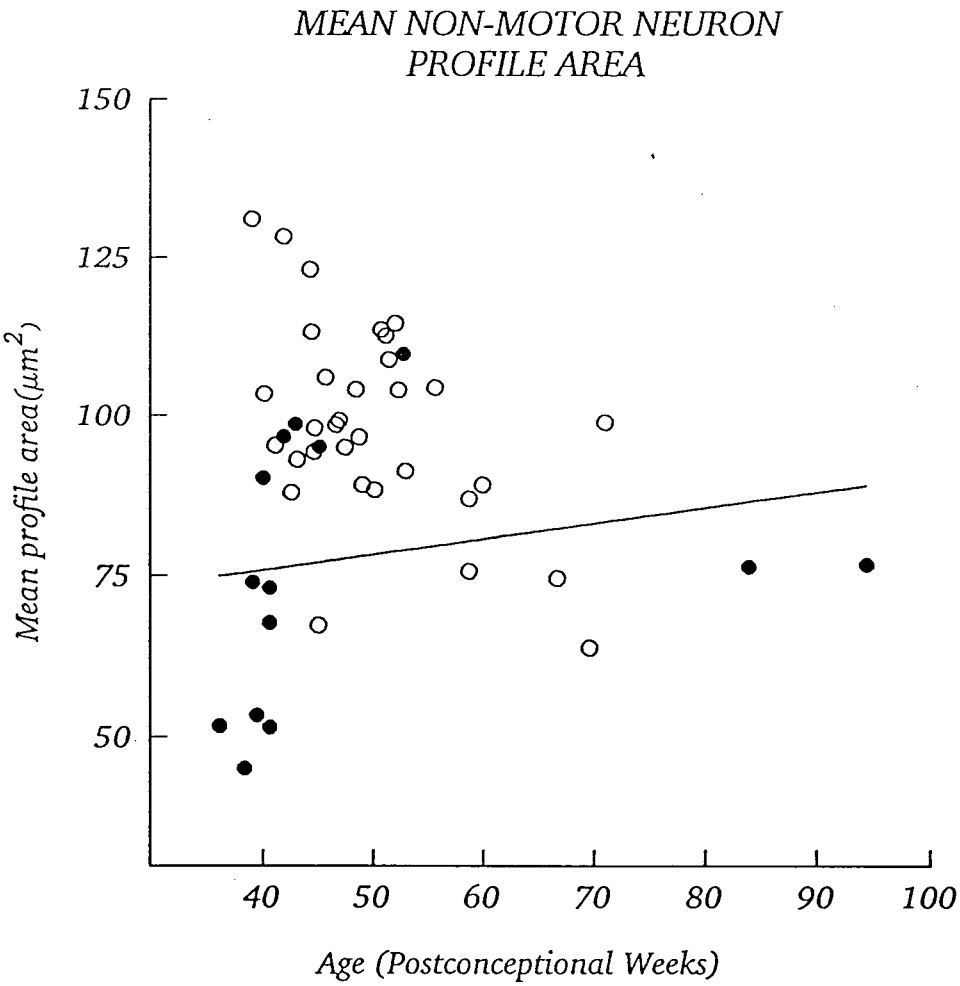


Figure 2.14. Mean profile area for cell bodies of non-motor neurons in the DMV of SIDS cases (open circles) and of control cases (filled circles) as a function of age in postconceptional weeks. Regression line is indicated for control data (solid line).

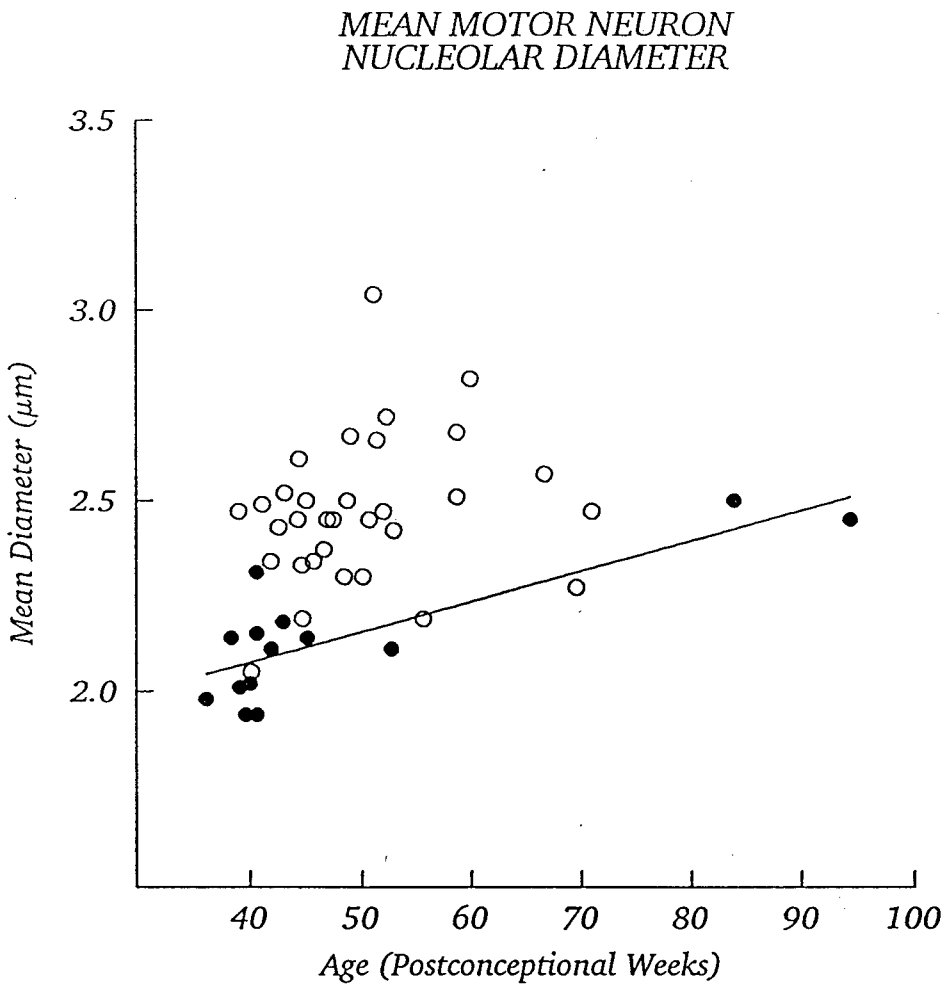
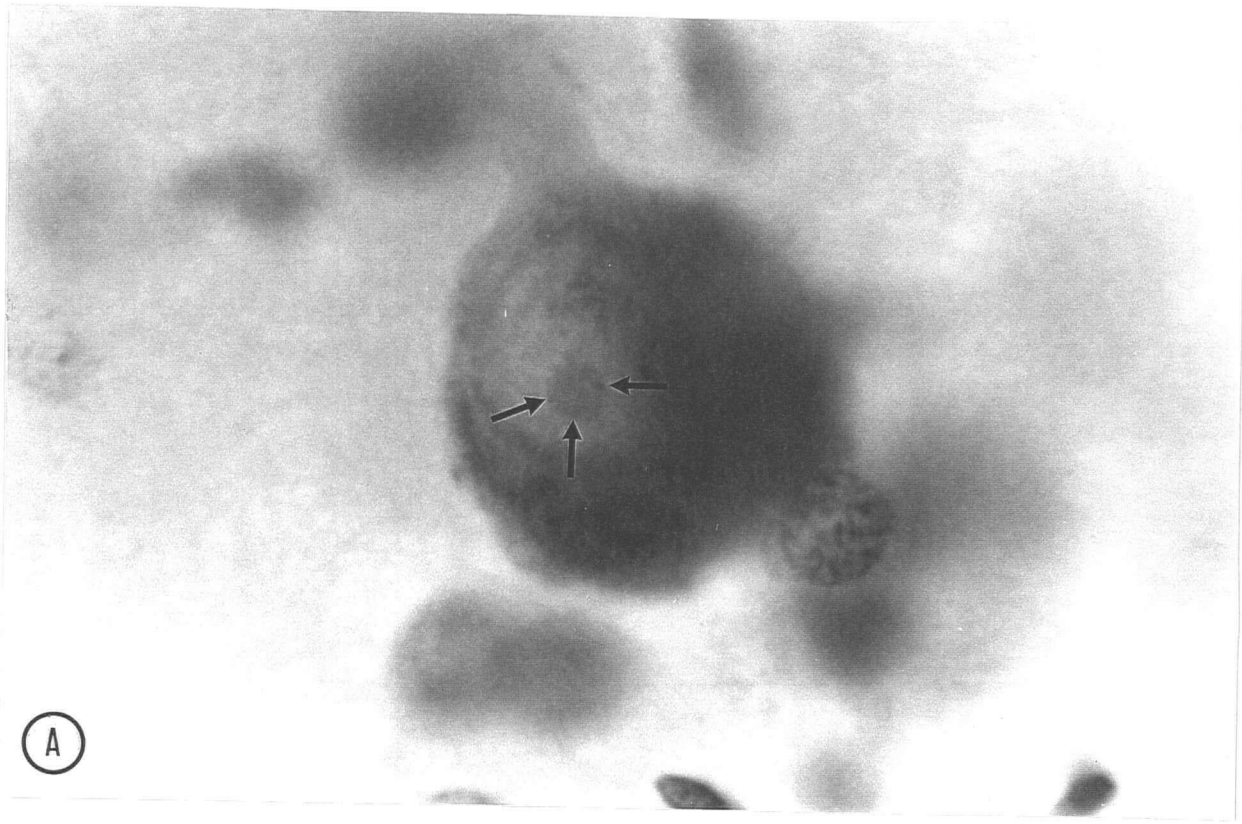


Figure 2.15. Mean nucleolar diameter of motor neurons in the DMV of SIDS cases (open circles) and of control cases (filled circles) as a function of age in postconceptional weeks. Regression line is indicated for control data (solid line).

Figure 2.16. Nucleolus of a motor neuron of the dorsal motor nucleus of the vagus in a SIDS victim at 41.1 postconceptional weeks of age (A) and a control case at 40 postconceptional weeks age (B).Note the presence of paranucleolar inclusion bodies (arrows) adjacent to the nucleolus in the motor neuron of the SIDS victim.Nissl stain (thionin). Bar=1μm.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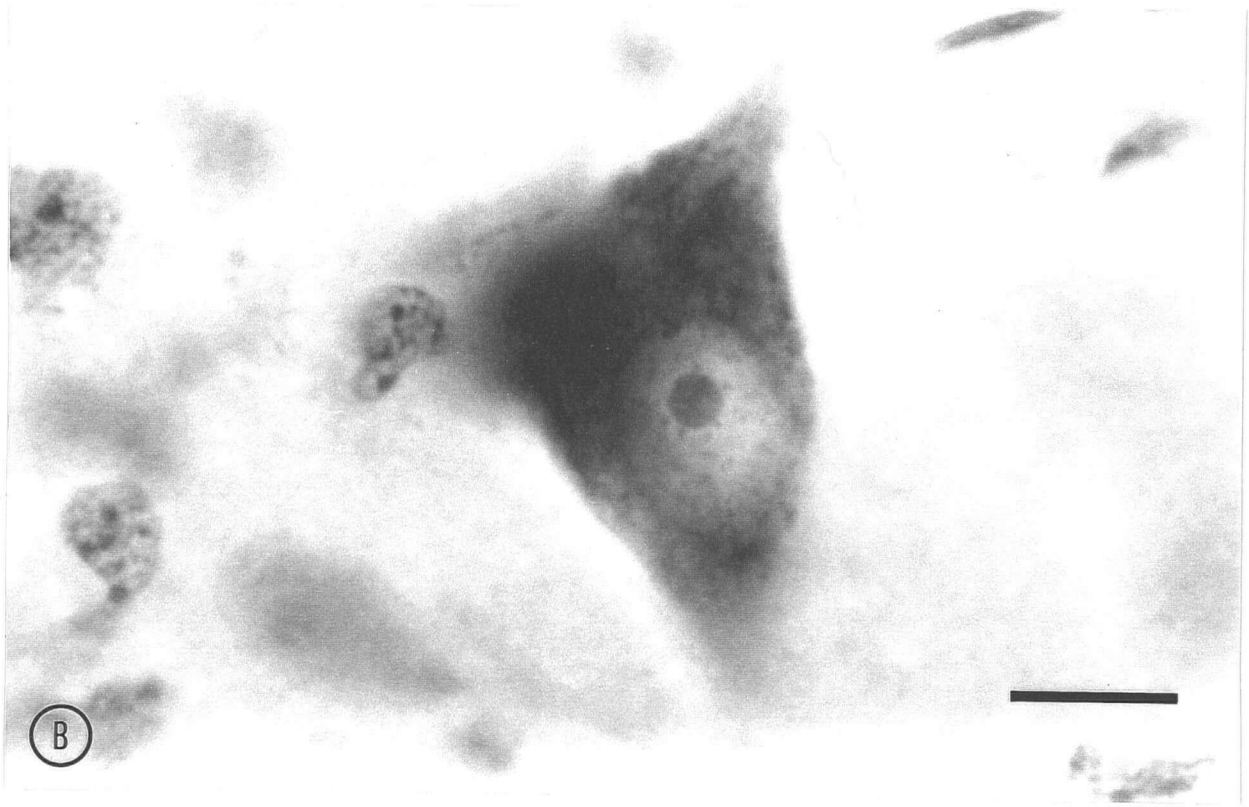
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Chapter 3.

The Role of Insulin-like Growth Factor I in the Postnatal Development of the Dorsal Motor Nucleus of the Vagus and the Hypoglossal Nucleus

3.1. Introduction

3.1.1 An overview of insulin-like growth factor-I

Insulin-like growth factor-I (IGF-I) is a monomeric peptide, which belongs to the insulin superfamily. During development, it is believed to play a crucial regulatory role in the growth and development of the central nervous system. IGF-I, its receptor, and its binding proteins, IGFBP1-6, are expressed early in development. Peak IGF-I mRNA expression has been shown to occur in the first two postnatal weeks in rodents (Bartlett et al., 1991; Bach et al., 1991).

Mounting evidence suggests IGF-I acts as a mitogen (Bartlett at al., 1991; for review see D'Ercole et al., 1996). Studies using cultured neuronal tissue have shown that the addition of IGF-I to the culture media results in stimulation of mitosis, neurite outgrowth, and induction of oligodendrocyte differentiation (Bondy, 1991). Observations of transgenic mice that overexpress IGF have shown a

significant increase in brain weight and size (Matthews et al., 1988) without gross anatomic abnormalities. It is thought that this increase is due to an increase in neuron number (Behringer et al., 1990), an increase in myelination (Ye et al., 1995) and an increased density of myelinated axons (Carson et al., 1993).

Mice that have an induced disruption of the IGF-I gene or the IGF-I receptor gene, exhibited significant brain growth retardation and abnormalities in the nervous system (Baker et al., 1993; Liu et al., 1993). Reducing the amount of useful IGF-I by inducing the ectopic expression of IGF-I binding proteins showed similar results (D'Ercole et al., 1994). These studies that increase or decrease the amount of available IGF *in vivo* show unequivocally that IGF-I has an important effect on the growth and development of the brain.

3.1.2. Central nervous system localization

It appears that IGF-I affects the development of most regions in the brain. Studies employing *in situ* hybridization histochemistry have found that IGF-I is expressed throughout the CNS. However, these studies showed considerable regional variation in the level of expression. Expression was found to be highest in areas where neurogenesis still persisted postnatally (Bartlett et al., 1991). These areas included the cerebellum, olfactory bulb, the hippocampal complex, and the rhombic lip of the developing brainstem.

Less is known about the function of IGF-I in the brainstem. It has been reported that overexpressing IGF-I transgenic mice have significant increases in brainstem weight (Ye et al., 1996). Also, *in situ* hybridization histochemistry analysis

located IGF-I mRNA in long-axon projection neurons with the highest specificity in functionally related sensory and cerebellar projection neurons (Bondy, 1991). IGF type I receptor mRNA has been shown to be rather uniformly distributed in the brainstem, with regional variation reflecting differences in neuronal density. Transient IGF-I gene expression has been reported in discrete brainstem nuclei with peak gene expression occurring at markedly different ages in individual nuclei (Bondy, 1991; Bondy et al., 1992). The role of IGF-I in controlling the growth and development of specific cardiorespiratory nuclei in the brainstem remains to be determined.

In the present study, morphometric analyses of the dorsal motor nucleus of the vagus (DMV) and the hypoglossal nucleus (HN) were performed following increased expression of IGF-I during early postnatal development in the mouse. Increased IGF-I expression was achieved using transgenic mice carrying the IGF-II/IGF-I transgene (IGF-II/I). Compared to normal littermate controls, the brains of IGF-II/I transgenic mice exhibit overgrowth beginning from the second postnatal week (Ye et al., 1996). Regional increases in brain weight have been reported in the brainstem, diencephalon, hippocampus, cerebellum and cerebral cortex. By postnatal day 50 cerebellar weight and DNA content were increased by 82% and 20%, respectively. The concentration of IGF-I in the cerebellum has been reported to be increased by 112% over littermate controls by 35 days of age (Ye et al., 1996), with significant increases in the number of granule cells and Purkinje neurons. Behavioural studies remain to be performed on these IGF-II/I transgenic mice. However, upon simple examination, they do not exhibit obvious behavioral

abnormalities, and they appear to thrive into adulthood. In the present study, the total volumes of the DMV and hypoglossal nucleus were compared in transgenic mice and normal littermate controls on postnatal day 35. In addition, the N_V and total number of neurons as well as the mean neuronal profile areas were determined in these groups. This analysis was designed to investigate the extent to which elevated levels of IGF-I in the brainstem during early postnatal development would produce developmental abnormalities in the DMV and hypoglossal nucleus similar to those seen in SIDS.

3.2. Materials and methods

3.2.1. Generation of transgenic mice

Generation of the IGF-II/I transgenic mice and the construction of the fusion gene were performed at the Transgenic Mice Facility of the program in Molecular Biology and Biotechnology at the University of North Carolina at Chapel Hill. IGF-II/I transgenic mice (n= 3) and normal littermate controls (n= 4) at 35 postnatal days of age were provided by Dr. A.J. D'Ercole (Department of Pediatrics, University of North Carolina at Chapel Hill). Techniques and procedures for the generation of these mice have been described in detail (Ye et al., 1996). Briefly, a 5.7 kb fragment of the 5' mouse IGF-II gene, containing exon 1, exon 2, and exon 3, was linked to a human IGF-I (hIGF-I) cDNA. This was in turn fused to the 3' flanking region of the human growth hormone (hGH) gene providing polyadenylation signals and sites. A 6.9 kb fusion gene fragment was excised from plasmids and purified. Classical microinjection technology was performed. Southern blot analysis was

performed, which allowed the identification of one line of mice expressing the transgene. The line of mice carrying the IGF-II/IGF-I transgene was bred as heterozygotes. Using tail genomic DNA, each offspring was identified as transgenic or normal littermate by PCR analysis.

3.2.2. Histological preparation

Mice were anaesthetized by an intraperitoneal injection of sodium pentobarbital (80 mg/kg) on postnatal day 35 and perfused through the ascending aorta, using a fixative solution containing 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The perfusion pressure was matched to the systolic blood pressure of the mouse at this age (100-110 mm Hg). The brains were removed and the brainstems, with attached cerebelli, were detached from the cerebral hemispheres. Serial frozen sections (30 µm in thickness) were cut in the transverse plane throughout the entire length of the brainstem and cerebellum. Every second section in the series was then mounted on chrome alum gelatin-coated glass slides and stained for Nissl substance using 0.1% aqueous thionine in acetate buffer (pH 3.7).

One transgenic brain and one normal littermate control brain were prepared for electron microscopy. Slabs of approximately 0.5-1.0 mm thickness were cut in the transverse plane through the length of the brainstem. The slabs were washed in buffer and postfixed in 1% buffered osmium tetroxide for 12 hours. They were then washed in acetate buffer, stained with 2% aqueous uranyl acetate for 1 hour, and dehydrated in ascending grades of ethanol. After dehydration, the sections

were equilibrated in propylene oxide and then embedded in Epon. Large semithin sections were cut at 0.5μm in approximately the frontal plane, mounted on glass slides and stained with 1% toludine blue in 0.4% sodium borate. In light microscopy a region containing the DMV and adjacent HN was identified on these semithin sections and the block was trimmed to contain only the DMV and a portion of the HN. Ultrathin sections of silver-gray interference color (60nm) were cut and mounted on #200 square mesh grids and stained with lead citrate. All histological specimens were coded to prevent bias during the morphometric analyses.

3.2.3. Morphometric analysis

Total volume of the DMV and the HN was measured in mm^3 using the serial Nissl sections through the brainstem. The caudal extent of both the DMV and the HN was defined as the most caudal section containing three or more motor neurons at the caudal limit of the medulla. The rostral extent of both the DMV and the HN was defined as the disappearance of clustered motor neurons nearing the pontomedullary junction. Volumes were then determined using the serial Nissl sections at a final magnification of X265. The morphometric analysis was performed following procedures previously reported (O'Kusky and Norman, 1992; O'Kusky et al., 1995; O'Kusky and Norman, 1995). Briefly, the area of each DMV and HN was measured in mm^2 on individual sections using a digitizing tablet of an image analysis system (Bioquant System IV, R&M Biometrics, Nashville, TN), following the boundary criteria of Paxinos and Watson (1986). The total volumes of the DMV and the HN were calculated from the following equation:

$$V = \sum A \times T \times P,$$

where $\sum A$ is the sum of the area measurements, T is the thickness of the section, and P is the periodicity of the sample. In this case, the thickness of the sections was 30 μm, with a periodicity of 2. Volumes were determined bilaterally for both the DMV and HN in each animal. Total length of the DMV and the HN was calculated using the formula:

$$L = \sum N \times T \times P,$$

where $\sum N$ is the total number of sections throughout the nucleus.

The numerical density of motor and non-motor neurons were determined using the Abercrombie method (Abercrombie, 1946). Four sections at both the rostral and caudal limits of the series were excluded. Counts were performed on every other section from the intervening sections. At a final magnification of X2,645, neurons were counted when a clear and distinct nucleolus was observed. The numerical density of neurons (N_V) was calculated as the number of neurons per mm^3 using the following equation:

$$N_V = N_A / (D + T)$$

Where N_A is the number of neuronal profiles per unit area of section, D is the mean nucleolus diameter, and T is the thickness of the section. Data were collected separately for motor neurons and non-motor neurons. Motor neurons were identified as having substantial amounts of intensely stained and clumped Nissl substance, larger cell body profile areas, and multipolar shape. Non-motor neurons were identified by pale-staining and uniformly distributed Nissl substance, smaller cell body profile areas, and round bipolar shape. The total numbers of motor and

non-motor neurons were determined in the DMV and HN from estimates of N_V and nuclear volume. In each animal the N_V and total number of neurons were determined bilaterally for both the DMV and HN.

The mean profile area for motor and non-motor neurons were determined at a magnification of X2,645. Using the Bioquant digitizing system, the boundaries of the neuron at a plane of focus that contained a clear and distinct nucleolus, were traced and the area measured in μm^2. Estimates of the mean profile areas were based on measurements of 100 motor neurons and 60 non-motor neurons in the DMV and HN of each animal. These neurons were randomly chosen evenly throughout the caudal-rostral extents and medial-lateral positions to avoid any spatial size differences.

The statistical significance of differences between IGF-II/I transgenic mice and littermate controls of age was determined using Student's t-test.

3.3. Results

The morphological appearance of the brain was comparable in the two groups at 35 days of age. No gross malformations, atrophy, or gliosis were apparent in transgenic mice when compared to controls.

3.3.1. Dorsal motor nucleus of the vagus

Boundaries of the DMV were reliably determined by the presence of medium sized motor neurons with substantial amounts of Nissl substance (Fig. 3.1). The motor neurons were easily distinguished from the non-motor neurons due to their

larger diameter and dense accumulation of Nissl substance. The neurons of the DMV were distinguished from the ventral lying hypoglossal neurons by their smaller size and rounder shape, and from the lateral reticular neurons and dorsal solitarius neurons by their larger size and increased Nissl substance. No gross morphological differences were seen in the DMV between the transgenic mice and the control mice.

Results of the morphometric analyses for the DMV are presented in Table 3.1. The total volume of the DMV was found to be approximately 0.02155 mm^3 in controls and approximately 0.03957 mm^3 in the transgenic mice. There was a significant increase in the volume of the DMV in transgenic mice, when compared to controls (84%, t= 17.040, df= 12, P< 0.0001). Representative sections through the medulla of IGF-II/I transgenic mice and controls are illustrated in Figure 3.1 for comparison. Upon further examination, it was found that this increase was due to both a 49% increase in mean area per section (t= 6.781, df= 12, P< 0.001) and a 23% increase in length (t= 4.114, df= 12, P< 0.01).

The N_v of all neurons in the DMV was significantly less in transgenic mice than in controls (16%, t= 2.264, df= 12, P< 0.05). Despite the decreased N_v, the total number of neurons in the DMV of transgenic mice was significantly greater than in controls (56%, t= 6.158, df= 12, P< 0.001). The N_v of motor neurons was significantly decreased in transgenic mice when compared to controls (18%,t= 2.318, df= 12, P< 0.05), although the N_v of non-motor neurons did not differ significantly (t= 0.501, df=12, P> 0.05). Total number of motor and non-motor neurons in transgenic mice, however, both displayed significant increases. In

transgenic mice the motor neurons were found to have a 49% increase ($t = 4.064$, $df = 12$, $P < 0.001$) and the non-motor neurons showed a 66% increase ($t = 6.695$, $df = 12$, $P < 0.001$), when compared to littermate controls.

The mean profile area of motor neuron cell bodies was significantly greater in transgenic mice than in controls (35%, $t = 7.948$, $df = 12$, $P < 0.001$). In addition, the mean profile area of non-motor neurons in transgenic mice was significantly greater than in controls (15%, $t = 2.310$, $df = 12$, $P < 0.05$), although the magnitude of the difference was less than for motor neurons.

Mean diameter of the nucleolus in motor neurons was found to be 2.30 ± 0.04 µm in transgenic mice and 1.90 ± 0.06 µm in controls, demonstrating a significant increase in transgenic mice (21%, $t = 5.644$, $df = 12$, $P < 0.001$). However, the mean nucleolar diameter for non-motor neurons did not differ significantly between transgenic (1.32 ± 0.08 µm) and control (1.25 ± 0.04 µm) mice at day 35 ($t = 0.808$, $df = 12$, $P > 0.05$).

3.3.2. Hypoglossal Nucleus

Boundaries of the hypoglossal nucleus were obvious due to the inclusion of large, multipolar, darkly staining motor neurons (Fig. 3.1). These were easily distinguished from the dorsally located DMV neurons, the lateral reticular neurons, and the ventrally located nucleus of Roller by size and presence of Nissl substance. Motor neurons were easily distinguished from the non-motor neurons by their larger profile size and darkly staining cytoplasm (Fig. 3.3)

Results of the morphometric analyses for the hypoglossal nucleus are

presented in Table 3.2. The mean volume in the transgenic mice was found to be 0.09627 ± 0.00302 mm^3, whereas the average volume in the control mice was 0.07454 ± 0.00271 mm^3. This is a significant increase of 30% in transgenic mice (t= 5.611, df= 12, P< 0.001). Representative sections through the medulla of IGF-II/I transgenic mice and controls are illustrated in Figure 3.1 for comparison. Unlike the DMV, the hypoglossal was found to have a sizeable increase in mean section area (28%, t= 5.455, df= 12, P< 0.001), but the length of the nucleus did not differ between subjects (t= 0.582, df= 12, P> 0.05).

The N_v of all neurons in the hypoglossal nucleus was found to be significantly lower in the transgenic mice when compared to control mice (13%, t= 2.069, df= 12, P< 0.05). The total number of neurons in the HN did not differ significantly between transgenic mice and controls (t= 1.214, df= 12, P> 0.05). The N_v of motor neurons was significantly less in transgenic mice (13%, t= 2.481, df= 12, P< 0.05), while the N_v of non-motor neurons did not differ significantly (t= 0.672, df= 12, P> 0.05). The total number of neurons in the HN did not differ significantly for either motor (t= 1.654, df= 12, P> 0.05) or non-motor (t= 0.452, df= 12, P> 0.05) neurons.

The mean profile area of motor neuron cell bodies in the hypoglossal nucleus was found to be significantly larger in the transgenic mice when compared to control mice (22%, t= 3.017, df= 12, P< 0.01). However, the mean profile area for non-motor neurons did not differ significantly between transgenic mice and controls (t= 2.062, df= 12, P> 0.05).

In transgenic mice, the mean diameter of the nucleolus did not differ

significantly from that of controls in either motor neurons ($t= 2.039$, $df= 12$, $P> 0.05$) or non-motor neurons ($t= 0.671$, $df= 12$, $P> 0.05$).

An interesting observation in both the DMV and the hypoglossal nucleus was made while measuring the diameter of the nucleolus. There appeared to be an increased size and preponderance of small dense bodies adjacent to the nucleolus in the transgenic mice, when compared to the control mice (Figs. 3.2, 3.3, 3.4, 3.5, and 3.6). In the DMV of transgenic mice (Fig. 3.2A), the nucleus of a large motor neuron can be seen to contain two densely stained spherical bodies adjacent to the nucleolus in apparently bilateral orientation. In controls (Fig. 3.2B) these sperical bodies were not readily apparent. In the transgenic mice, these paranucleolar inclusions were also observed in the nuclei of non-motor neurons. These nucleolar inclusion bodies were occasionally observed in the control mice, but they were considerably reduced in size with less intense staining. In the HN of transgenic mice (Fig. 3.3) similar inclusions were frequently observed in both motor and non-motor neurons. As observed in the DMV, the largest of these inclusions (e.g. reaching diameters of 1.0-1.5 μm) in neurons of the HN frequently were found at opposite ends of the nucleolus (Fig. 3.4A). In the HN of control cases, small particulates of stained material could be observed around nucleoli (Fig. 3.4B) in many neurons. However, these were barely at the limits to resolution for the light microscope and seldom approached the size and staining intensity of the sperical inclusions seen in transgenic mice. Electron microscopy revealed that these paranucleolar inclusion bodies were consistent in texture and granularity to the pars fibrosa of the nucleolus. In control neurons of the HN (Fig. 3.5A) and the DMV (Fig.

3.5B), a uniform distribution of nuclear chromatin was observed along with some variation in the size of nucleoli. The nucleoli consisted of the pars fibrosa, which is made up of fibrillar chromatin, interspersed within pars granulosa, which forms a matrix enclosing the pars fibrosa. Occasionally, the contents of the pars fibrosa can be seen escaping the matrix of the nucleolus (Fig 3.5) and appears to form a perinucleolar mass of fibrillar chromatin beside the nucleolus. The size and frequency of this spherical fibrillar material is consistent with the small paranucleolar inclusion bodies seen in light microscopy.

Electron microscopy of nucleoli in the motor neurons of DMV (Fig. 3.6A) and HN (Fig. 3.6B) of transgenic mice revealed a different appearance than that of control mice. In many cases there appeared to be an enlargement of the pars fibrosa and large accumulations of perinucleolar chromatin (Fig. 3.6A). These masses were consistent with the paranucleolar inclusions observed in light microscopy. Many examples of massed perinucleolar chromatin, accumulated at either end of the nucleolus, were observed (Fig. 3.6B). These enlarged masses of perinucleolar chromatin had the appearance of coiled bodies, which usually appear as 0.3-0.8 μm aggregates of electron-opaque coiled fibrils resembling the cords of the nucleolar pars fibrosa.

3.4 Discussion

In the IGF-II/I transgenic mice, transgene expression has been detected as early as embryonic day 18 with increasing gene expression to a maximum at approximately 15-20 postnatal days (Ye et al., 1996). Tissue concentrations of IGF-

I continue to be significantly elevated throughout postnatal development into the adult animals (Ye et al., 1996). Morphometric analyses in the present study have shown a number of developmental abnormalities due to elevated levels of IGF-I in both the DMV and the HN. Transgenic mice displayed a 30% increase in the total volume of the HN, when compared to control littermate controls. The DMV, on the other hand, showed an 84% increase in volume. Interestingly, the increased volume of the HN was due to an increase in the girth (29%) of the nucleus, but not due to any lengthening along the caudal-to-rostral extent of the neuraxis. On the other hand, the increased volume of the DMV was due to both an increased girth (51%) and length (23%). This difference may be due to the effects of IGF on neuronal migration for neurons of the DMV during development. The differences could also be due to the larger relative increase in the volume of the DMV and the limited space available for expansion. The finding of increased volume in these IGF-I transgenic mice is consistent with the role of IGF-I in promoting neuron survival, neurite outgrowth and increased brain weight (Bondy, 1991; Matthews et al., 1988; D'Ercole et al., 1996).

Total number of all neurons in the DMV was found to be increased in the transgenic mice when compared to controls (56%). There was a 49% increase in motor neurons and a 66% increase in non-motor neurons. This finding is consistent with previous studies demonstrating increased numbers of neurons in whole brain (Behringer et al., 1990), cerebral cortex (Gutierrez-Ospina et al., 1997), and cerebellum (Ye et al., 1996). The mechanism for this increase must involve either increased neurogenesis or decreased neuron death during late prenatal and early

postnatal development. This increase in neuron number was not observed in the HN for either motor neurons or non-motor neurons. The differential effect in adjacent nuclei could be suggestive of neuronal growth factor specificity for neurons in these two nuclei, but it is more likely due to the differential timing of DMV development compared to the HN.

In the developing brainstem of the mouse, primitive neurons destined for both the DMV and HN are generated at embryonic day 9-10 in the germinal zone of the IVth ventricle (Taber Pierce, 1973). In the HN of the rat, naturally occurring neuron death has been shown to occur exclusively during prenatal development, with a loss of 35% of cells between embryonic day 16 and birth at approximately embryonic day 21 (Friedland et al., 1995). This apoptotic neuron death occurs remarkably early in the HN, when compared to the death of motor neurons in the ventral horn of the spinal cord in rodents at postnatal days 2-12 (Romanes, 1946). The time course of neuron death in the DMV has not been documented in either the mouse or the rat. However, it is most likely to occur during early postnatal development, following most other nuclei in the brainstem, diencephalon and telencephalon (for example, Heumann and Leuba, 1983; Alley, 1974). A relatively delayed development of the DMV, compared to the motor trigeminal, hypoglossal and facial nuclei, has been reported in human development (Nara et al., 1991). In IGF-II/I transgenic mice it would appear that the increased number of neurons in the DMV, but not in the HN, results from an antiapoptotic effect of elevated IGF-I. The elevated levels of IGF-I occur after birth in these transgenic mice (Ye et al., 1996), at a time when neuron death is already completed in the HN and presumably still

occurring in the DMV.

A previous study of IGF-II/I transgenic mice revealed a significant increase in the number of Purkinje cells (20%) and granule cells (82%) in the cerebellum (Ye et al., 1996). In these animals BrdU histochemistry provided no evidence for increased neurogenesis in the external granular layer of the cerebellum, leaving the authors to conclude that elevated IGF-I levels probably decreased neuron death (Ye et al., 1996). *In vitro* studies have shown that IGF-I increases the survival of neurons in culture. Torres-Aleman et al. (1992) have shown a dramatic 7-fold increase in survival of cultured cerebellar cells from postnatal day 15 rats when IGF-I was added to the culture medium. Similarly, these authors (Torres-Aleman et al., 1994) have shown an increase in neurite outgrowth as well as cell survival in cultured cerebellar cells with added IGF-I. D'Mello et al. (1993) reported that IGF-I inhibited apoptosis induced by low potassium levels in cultured cerebellar cells. Similar results have been found in the hypothalamus (Pons et al., 1991; Torres-Aleman et al., 1990a, 1990b; Pons and Torres-Aleman, 1992).

Because there was a lower numerical density of neurons in both the DMV and the HN, increased neuropil area must exist between the motor neurons, contributing to the increased volume. Previously, it has been reported that there is an increase in neurite outgrowth when neurons are subjected to increased levels of IGF-I (Torres-Aleman et al., 1994). IGF-I, therefore, could conceivably cause neurite outgrowth in these brainstem nuclei. Given that an increased complexity of dendritic and axonal arborizations would likely harbor increased numbers of synaptic contacts, further research is necessary to quantify the total number of

synapses in these transgenic mice as compared to littermate controls. This increased connectivity, due to extraneous or anomalous axonal projections within a brainstem nucleus, would most likely cause respiratory or cardiovascular abnormalities.

An increase in the mean profile area of motor neurons was found in both the DMV (35%) and the HN (15%) of the transgenic mice. This increased size of the cell body is likely indicative of the increased dendritic arborization. There was an increase in the mean profile area of non-motor neurons in the DMV, but not in the HN. Nucleolar diameter was increased in the motor neurons of both nuclei of the transgenic mice, as well as having the observed nucleolar inclusion bodies (Figs. 3.5 and 3.6). The increased diameter of the nucleolus suggests an increase in activity and production of ribonucleoproteins. There appeared to be an increase in the size and frequency of nucleolar inclusion bodies, which were indistinguishable from intranuclear coiled bodies (Padykula and Clark, 1981; Bouteille et al., 1982; Brasch and Ochs, 1992; Goessens, 1984). This suggests either metabolic hyperactivity or an increase in transcription of ribosomal mRNA and increased protein synthesis in response to a growth factor (for review see, Brasch and Ochs, 1992). Since these nucleolar inclusions were found in both the motor and the non-motor neurons, elevated IGF-I evidently exerts a generalized effect on developing neurons in these nuclei.

The overgrowth phenomena observed in the HN of these IGF-II/I transgenic mice were virtually identical to previous reports of morphological abnormalities in the HN of SIDS cases (O'Kusky and Norman, 1992; O'Kusky et al., 1995).

Unfortunately, the increased number of neurons in the DMV in IGF-II/I transgenics did not agree with developmental abnormalities found in SIDS (see Chapter 2), although many other variables of overgrowth were similar. If elevated IGF-I inhibits apoptosis, resulting in increased neuron number, then delaying the elevated levels of IGF-I until after the critical period of neuron death in a mouse would conceivably produce overgrowth more closely resembling that seen in the DMV in SIDS. Because timing of IGF-II/IGF-I transgene expression cannot be easily modified in this transgenic mouse, more controlled timing could be achieved by the application of exogenous recombinant IGF-I to the brainstem of a normal animal during early postnatal development. Furthermore, the magnitude of the increase in IGF-I can be varied through concentration of the growth factor and volume of the injection.

Table 3.1 Morphometric Variables for the Dorsal Motor Nucleus of the Vagus in IGF-I Transgenic Mice and Littermate Controls at 35 Postnatal Days of Age

Variable	Transgenic	Control
Volume (mm^3)	0.03957 ± 0.00087***	0.02155 ± 0.00060
Length (mm)	1.020 ± 0.010**	0.832 ± 0.044
Mean area per section (mm2)	0.0388 ± 0.0009***	0.0258 ± 0.0017
N_V (cells per mm^3)	77,511 ± 6,355*	92,779 ± 3,430
Motor neurons	58,613 ± 5,642*	71,901 ± 2,665
Non-motor neurons	20,031 ± 752	20,879 ± 1,514
Total number of neurons	3,099 ± 193***	1,992 ± 704
Motor neurons	2,304 ± 201***	1,547 ± 547
Non-motor neurons	746 ± 41***	445 ± 24
Mean cell profile areas (μm^2)		
Motor neurons	280.85 ± 3.71***	207.83 ± 7.51
Non-motor neurons	98.86 ± 4.92*	87.41 ± 2.51
Mean nucleolar diameter (μm^2)		
Motor neurons	2.30 ± 0.04***	1.90 ± 0.06
Non-motor neurons	1.32 ± 0.08	1.25 ± 0.04

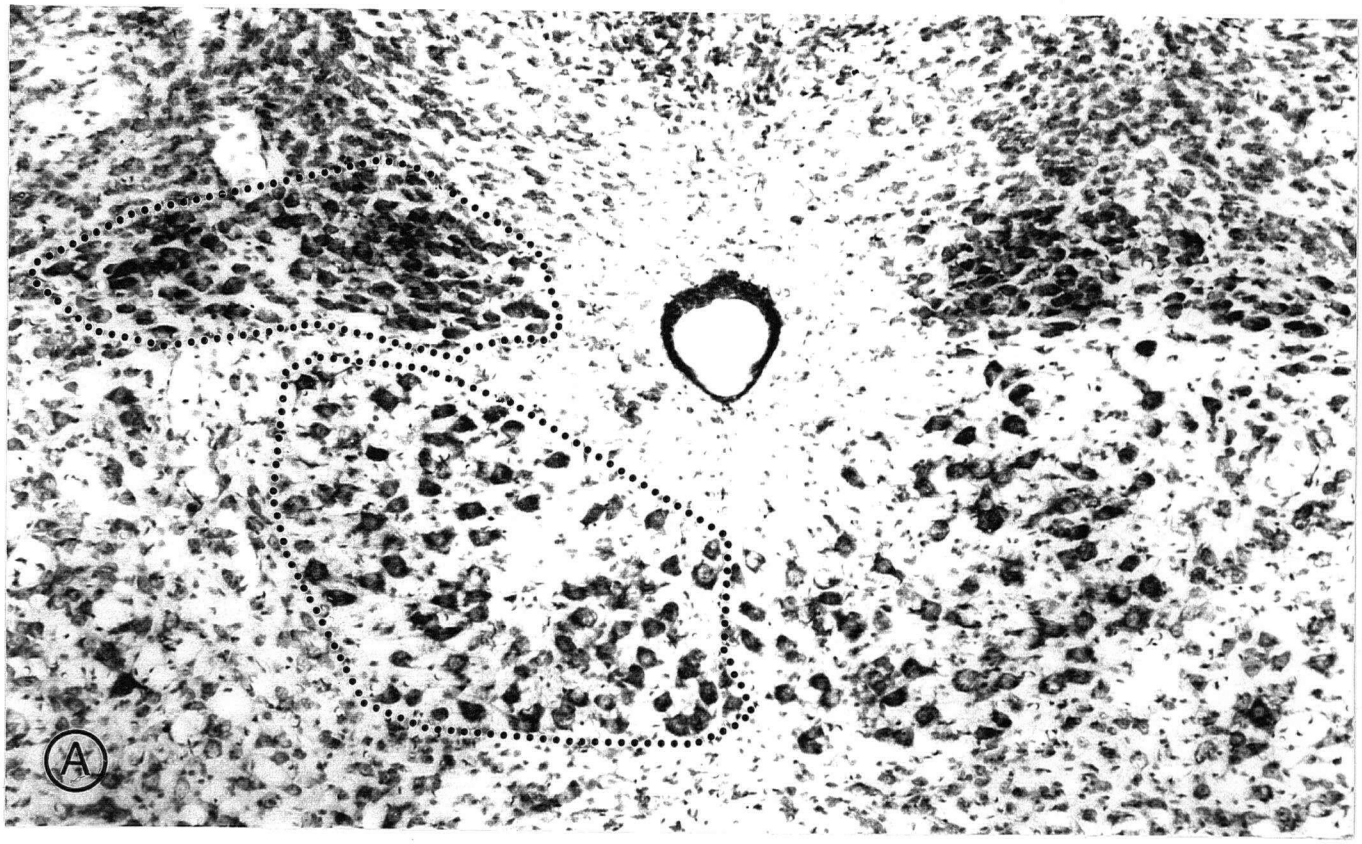
Values represent means ± S.E.M.
*** $P < 0.001$, compared to normal controls.
** $P < 0.01$, compared to normal controls.
* $P < 0.05$, compared to normal controls.

Table 3.2 Morphometric Variables for the Hypoglossal Nucleus in IGF-I Transgenic Mice and Littermate Controls at 35 Postnatal Days of Age

Variable	Transgenic	Control
Volume (mm^3)	0.0963 ± 0.0003***	0.0745 ± 0.0027
Length (mm)	1.090 ± 0.030	1.060 ± 0.050
Mean area per section (mm2)	0.0885 ± 0.0030***	0.0692 ± 0.0022
N_V (cells per mm^3)	21,417 ± 1,026*	24,690 ± 1,129
Motor neurons	17,854 ± 480*	21,373 ± 331
Non-motor neurons	3,562 ± 784	4,392 ± 421
Total number of neurons	2,071 ± 145	1,931 ± 92
Motor neurons	1,718 ± 73	1,599 ± 52
Non-motor neurons	351 ± 84	332 ± 43
Mean cell profile areas (μm^2)		
Motor neurons	421.16 ± 25.65**	348.54 ± 11.71
Non-motor neurons	168.90 ± 6.27	154.71 ± 4.66
Mean nucleolar diameter (μm^2)		
Motor neurons	2.41 ± 0.05 *	2.20 ± 0.09
Non-motor neurons	1.44 ± 0.02	1.41 ± 0.04

Values represent means ± S.E.M.
*** $P < 0.001$, compared to normal controls.
** $P < 0.01$, compared to normal controls.
* $P < 0.05$, compared to normal controls.

Figure 3.1. Dorsal motor nucleus of the vagus (top) and hypoglossal nucleus (bottom) outlined in a IGF-I transgenic mouse (A) and a littermate control mouse (B) at postnatal day 35. Note the increased area of nuclei in the transgenic mouse when compared to the normal littermate control mouse. Nissl stain (thionin). Bar=10μm.


A

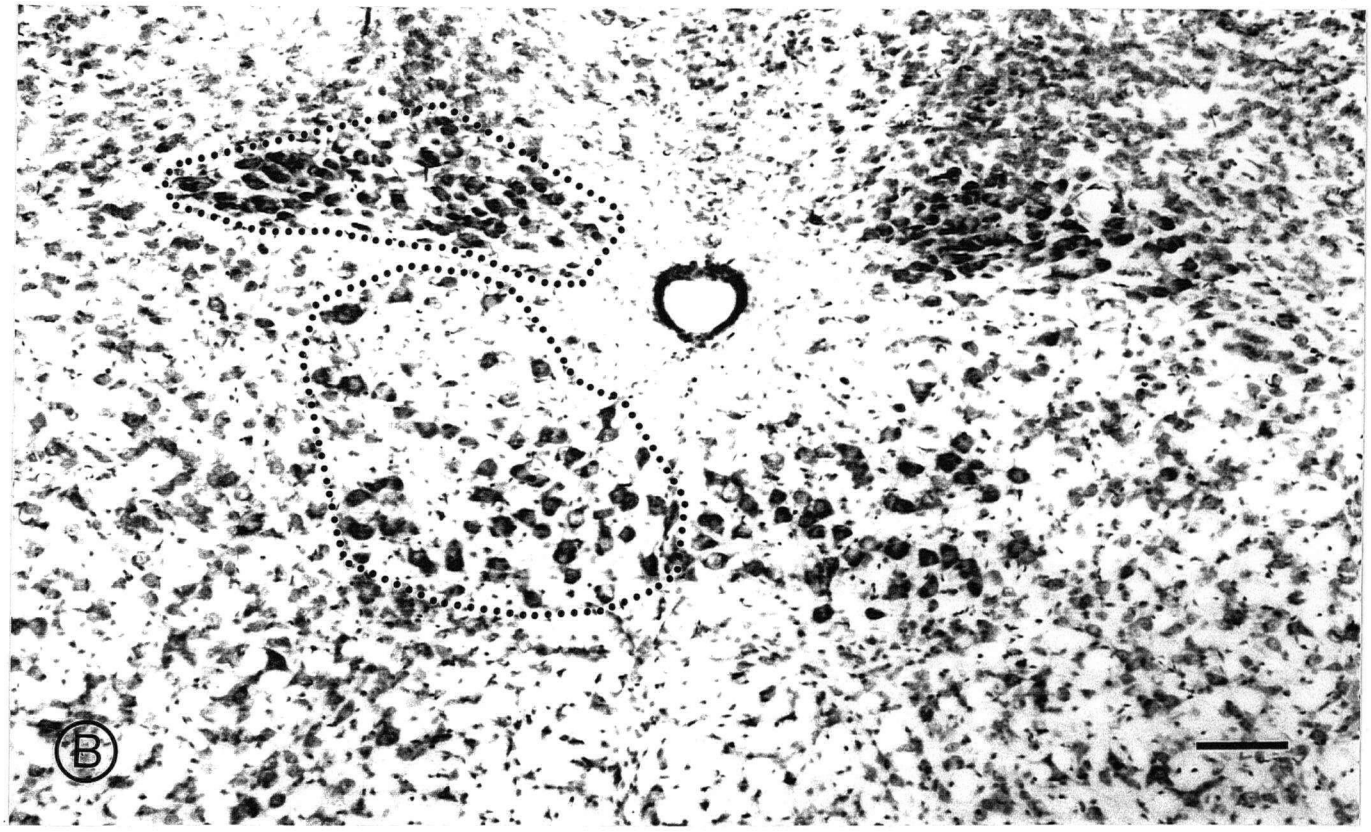
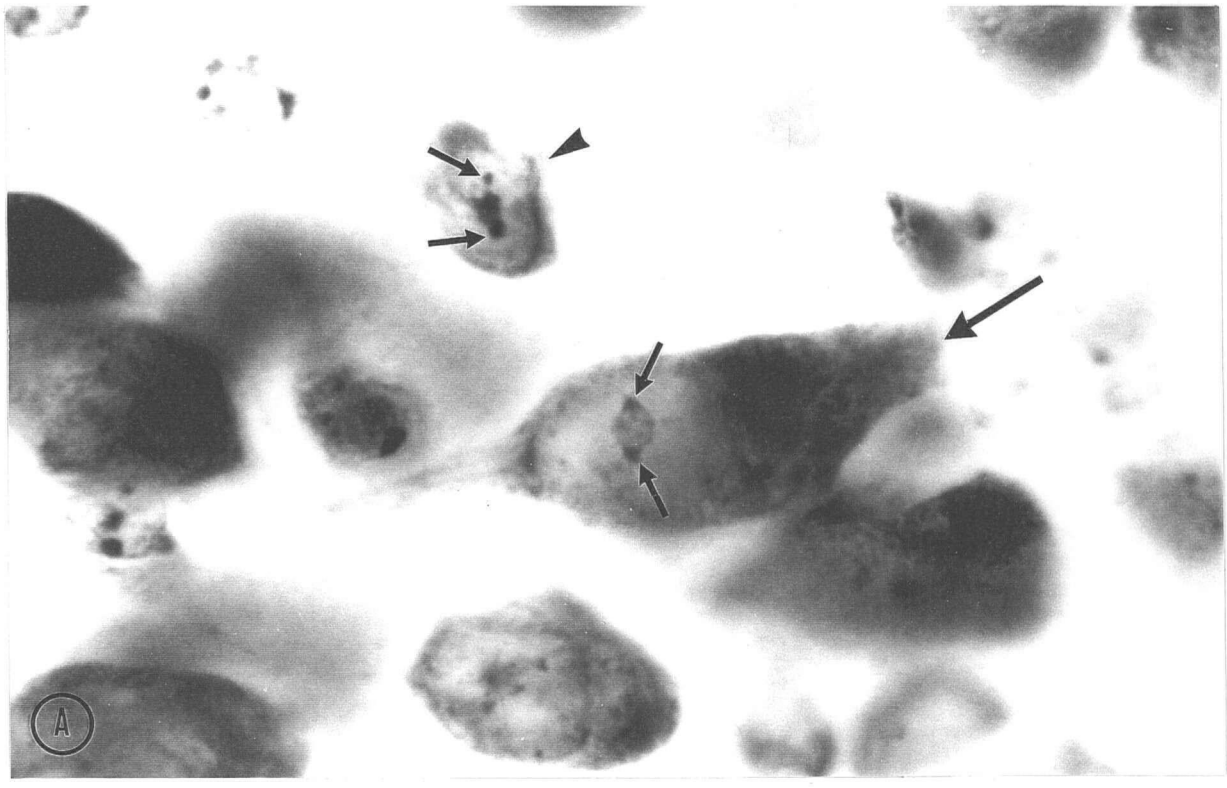

B

Figure 3.2. Neurons of the dorsal motor nucleus of the vagus in a IGF-I transgenic (A) and a littermate control (B) mouse at 35 postnatal days of age. The motor neurons (large arrow) are easily distinguished from the non-motor neurons(arrow head) by their larger profile area and their dense accumulation of Nissl substance. Note the dense nucleolar inclusion bodies situated bilaterally adjacent to the nucleolus (small arrows) in both cell types of the transgenic mouse. Nissl stain (thionin). Bar=1μm.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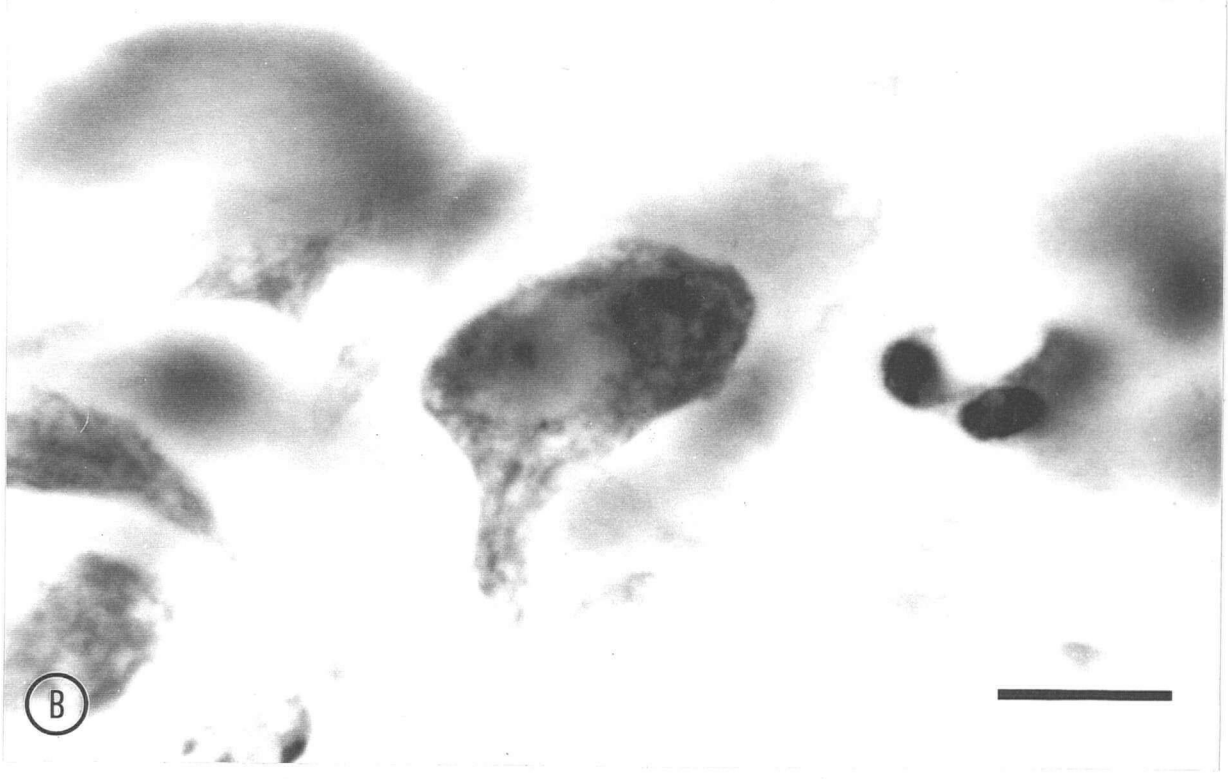

B

Figure 3.3. Neurons of the hypoglossal nucleus in an IGF-I transgenic mouse at 35 postnatal days of age. The motor neurons (large arrow) are easily distinguished from the non-motor neurons(arrow head) by their larger profile area and their dense accumulation of Nissl substance. Note the dense nucleolar inclusion bodies adjacent to the nucleolus (small arrows) in both cell types of the transgenic mouse. Nissl stain (thionin). Bar=1μm.

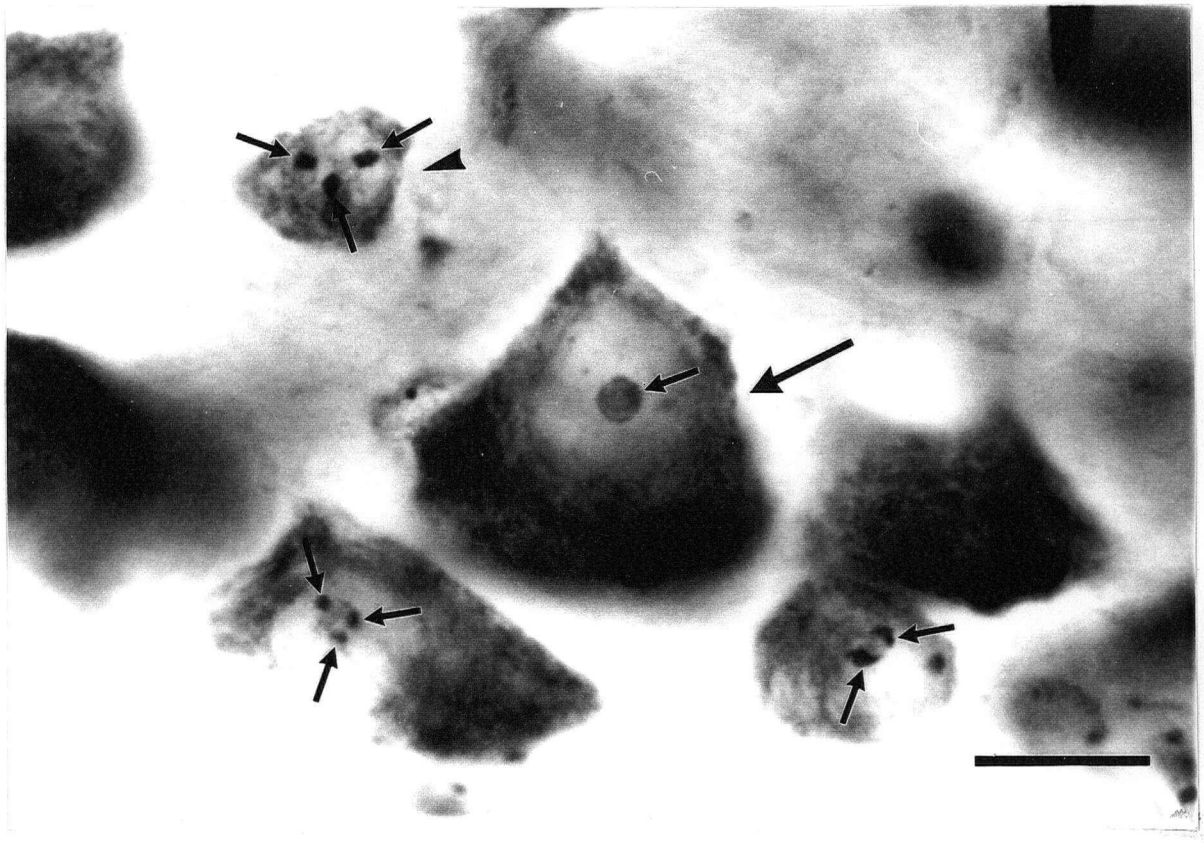
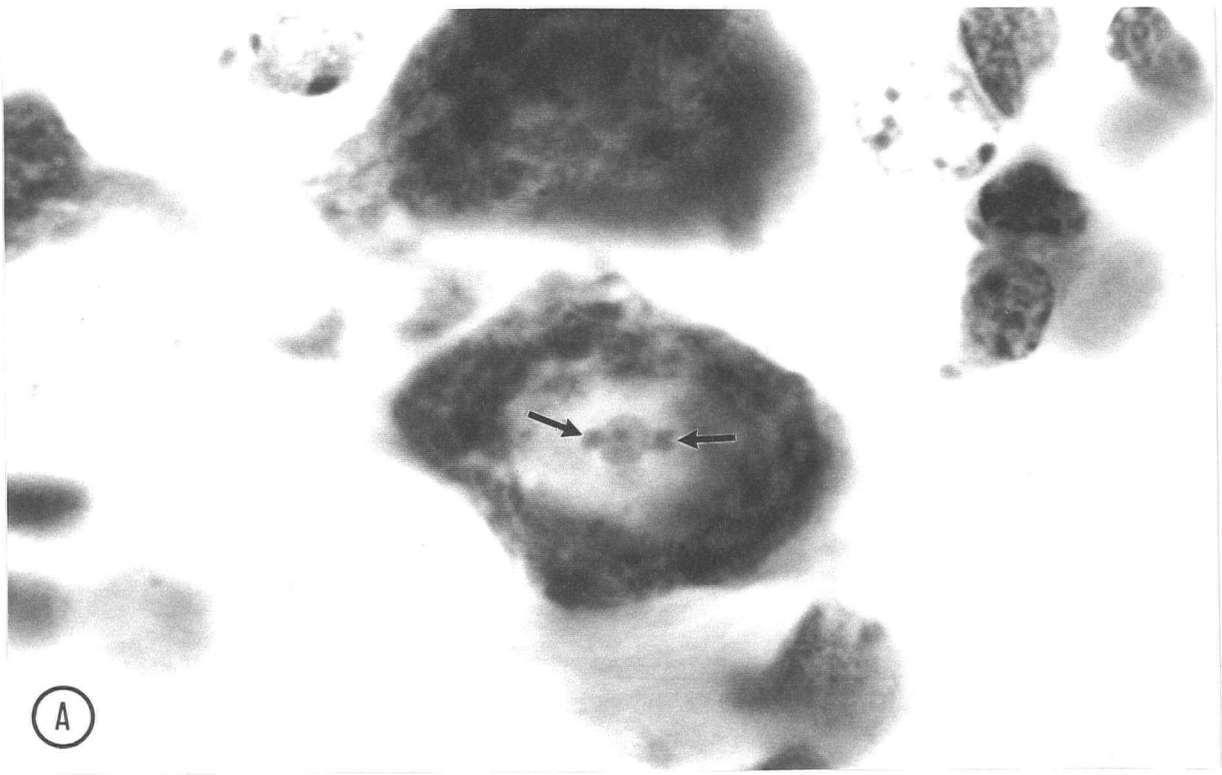


Figure 3.4. Neurons of the hypoglossal nucleus in an IGF-I transgenic mouse (A) and a normal littermate control (B) at 35 postnatal days of age. Note the dense nucleolar inclusion bodies situated bilaterally adjacent to the nucleolus (arrows) in the neuron of the transgenic mouse. Nissl stain (thionin). Bar=1μm.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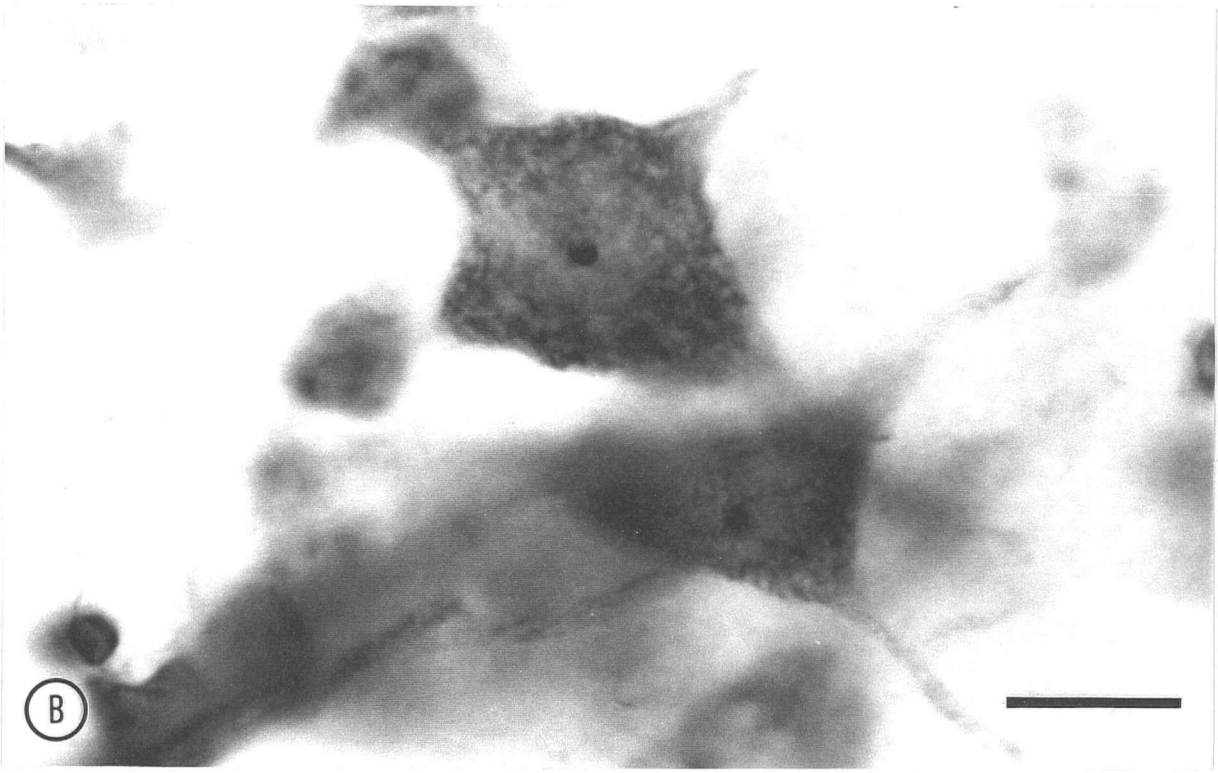
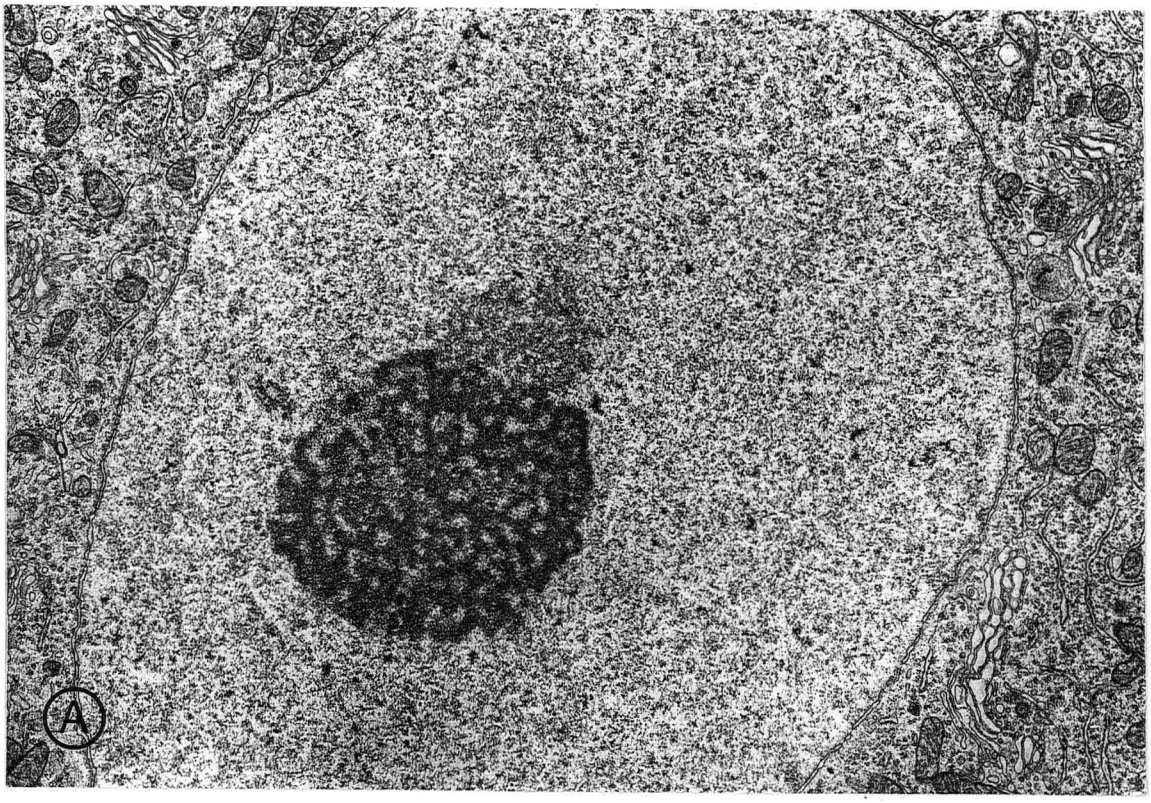

B

Figure 3.5. Electron micrograph of a motor neuron in the hypoglossal nucleus (A) and a motor neuron in the dorsal motor nucleus of the vagus (B) from a normal mouse at 35 postnatal days of age. Note the fibrillar mass, which appears to be consistent with the pars fibrosa, escaping from the matrix of the pars granulosa. Bar=200 nm.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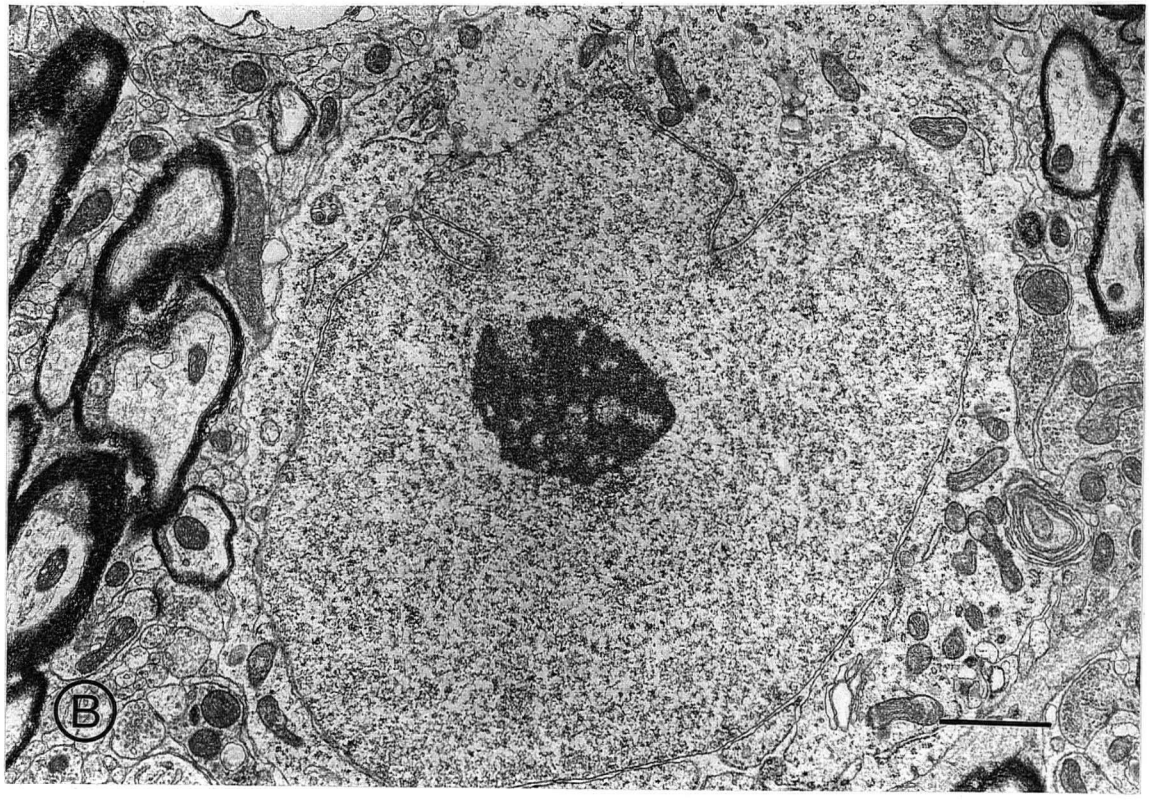
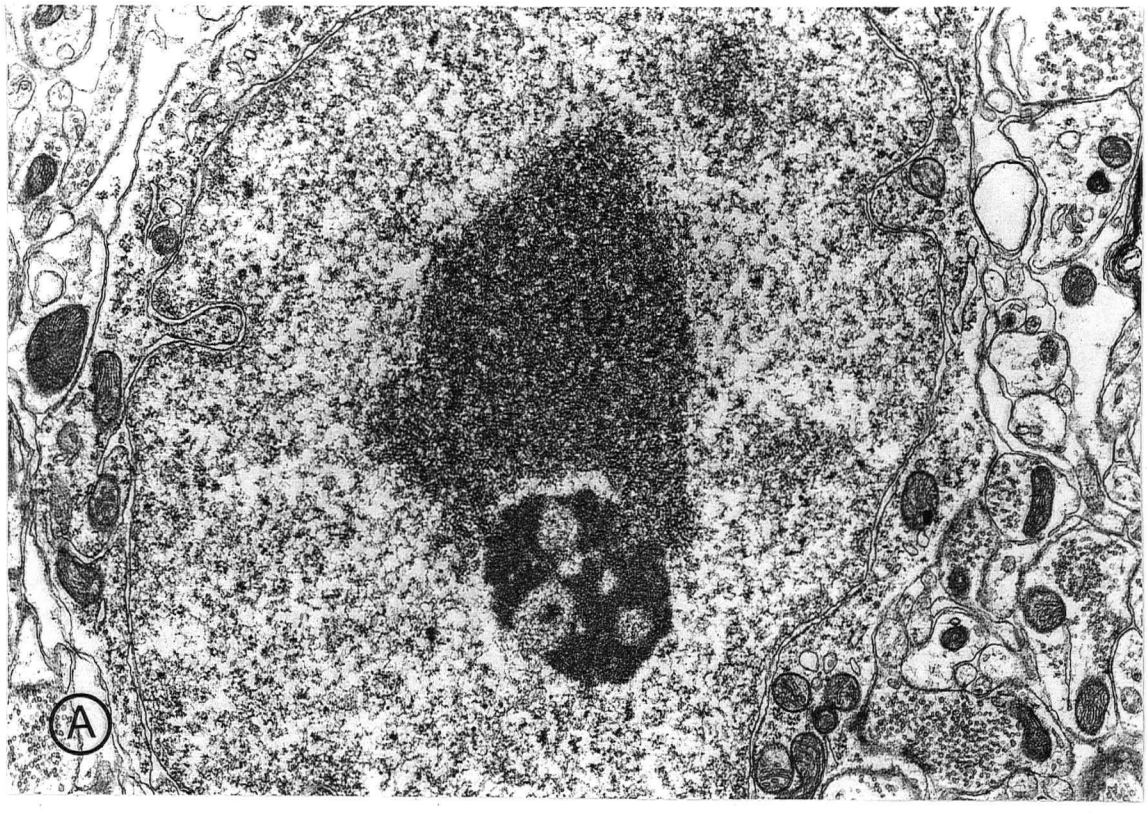

B

Figure 3.6. Electron micrograph of a motor neuron in the hypoglossal nucleus (A) and a motor neuron in the dorsal motor nucleus of the vagus (B) from a IGF-I transgenic mouse at 35 postnatal days of age. Note the large fibrillar mass, which appears to be consistent with the pars fibrosa, escaping from the matrix of the condensed pars granulosa. Note also the bilateral orientation of the perinucleolar masses adjacent to the nucleolus (B). Bar=200 nm.


A


B

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Chapter 4

Synapse Elimination During Normal Development of the Dorsal Motor Nucleus of the Vagus of the Rat

4.1. Introduction

Normal synaptogenesis is characterized by two distinct phases during development (for reviews, see Cowan et al., 1984; Purves and Lichtman, 1980; Wolf and Missler, 1993). There is an initial phase of synapse proliferation, where the N_V of synapses increases. This value reaches a maximum at a characteristic age, and then decreases in a subsequent regressive phase to levels characteristic of the young adult. This overproduction of synapses has been termed synaptic overshoot (O'Kusky and Colonnier, 1982). Numerous studies have demonstrated overshoot in the cerebral cortex of rat (Aghajanian and Bloom, 1967; Markus and Petit, 1987) , cat (Cragg, 1975; O'Kusky, 1985; Winfield, 1983), monkey (Missler et al., 1993; O'Kusky and Colonnier, 1982; Rakic et al., 1986), and human (Huttenlocher et al., 1982; Huttenlocher and deCourten, 1987). The timing of elimination, however, varies between species. For example, synaptic density in the cerebral cortex is at a maximum value by postnatal day 30 in the rat, day 70 in the cat, by 4-6 months in the monkey, and 8-12 months in humans. Most studies have found that the magnitude of overshoot ranges from 30-50%.

The timing of the progressive and regressive phases of synaptogenesis in brainstem nuclei, which are involved in the control of cardiac and respiratory physiology, is largely unknown. Neuromuscular synaptogenesis in the rat has a progressive phase that occurs from embryonic day 16 until peak densities are achieved at birth (Redfern, 1970). The elimination of extraneous synapses is completed by postnatal day 15 (Redfern, 1970). In the hypoglossal nucleus of the rat, it has been shown that the N_V of synapses increases from birth to peak values at postnatal day 20, followed by a significant decrease to levels characteristic of the young adult by day 30 (O'Kusky, 1998). This pattern of overshoot was demonstrated for both asymmetric and symmetric contacts (O'Kusky, 1998). In the cerebral cortex of rat, however, peak synaptic densities are reached as late as day 30 (Markus and Petit, 1987; Aghajanian and Bloom, 1967). Although the ages at which peak densities occur are not known for cardiorespiratory nuclei in the medulla and pons, It would appear that synapse proliferation and elimination most likely follow a caudal-to-rostral gradient along the neuraxis.

The mechanism underlying synapse elimination and axonal remodeling is unknown. It is thought, however, that competition among redundant contacts for a trophic factor is involved (English and Schwartz, 1995; Cowan et al., 1984; Purves and Lichtman, 1980; Wolf and Missler, 1993). The role of synaptic activity has also been postulated as an elimination determinant. O'Brien et al. (1978), for example, showed that when electrical stimulation was applied to the sciatic nerve to increase activity, a more rapid elimination of neuromuscular synapses at the polyinnervated junction occurred.

The purpose of the present study was to investigate synaptogenesis in the dorsal motor nucleus of the vagus (DMV) of rats during normal postnatal development. Morphometric and stereological analyses were used to determine postnatal changes in the volume of the nucleus as well as in the N_V and total number of synapses from birth to day 30 and in young adults. It has been suggested that SIDS involves a failure to eliminate normally transient synapses on neurons in brainstem respiratory nuclei. In order to develop an animal model of SIDS in the rat, one would need to know the time course of the progressive and regressive phases of synaptogenesis in brainstem respiratory nuclei. The experimental application of exogenous neuronal growth factors to discrete brainstem nuclei, immediately prior to the age of peak synaptic densities, could conceivably prevent the normal elimination of synapses. Such an experimental preparation would permit detailed studies of the neuropathology and pathophysiology of cardiorespiratory instability.

4.2. Materials and Methods

4.2.1. Tissue preparation

Forty-two Sprague-Dawley rat pups and 6 adult rats were used in this study. Six litters were culled to 8 pups per litter at birth. Individual litters were housed in an air-conditioned room with a 12-hour light/12-hour dark cycle and fed a standard pelleted diet ad libitum Six rat pups were perfused on each of postnatal days 1, 5, 10, 15, 20, 25, or 30. The 6 adult rats were perfused at approximately postnatal day 120. Sodium pentobarbital (80mg/kg) was administered by intraperitoneal injection

for general anesthesia. Individual rats were perfused through the ascending aorta with a fixative solution containing 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Perfusion pressure was adjusted to match systolic blood pressure at each age (70-110 mm Hg). The brains were removed and placed in additional fixative at 4°C for 24 hours.

Four brains at each age were prepared for light microscopy. This included serial frozen sections cut at 30 µm in the frontal plane through the entire length of the brainstem. Every second section was mounted on a chrome alum coated glass slide and stained for Nissl substance using 0.1% thionin in acetate buffer (pH 3.7). The remaining two brains at each age were prepared for electron microscopy. Slabs of approximately 0.5-1.0 mm thickness were cut in the transverse plane through the length of the brainstem. The slabs were washed in buffer and postfixed in 1% buffered osmium tetroxide for 2.5 hours. They were then washed in acetate buffer, stained with 2% aqueous uranyl acetate for 1 hour, and dehydrated in ascending grades of ethanol. After dehydration, the sections were equilibrated in propylene oxide and then embedded in Epon. Large semithin sections were cut at 0.5µm in approximately the frontal plane, mounted on glass slides and stained with 1% toludine blue in 0.4% sodium borate. In light microscopy a prominent DMV was identified from these semi-thin sections and the block was trimmed to contain only the nucleus and the immediately adjacent peripheral tissue. Ultra-thin sections of silver-gray interference color (60nm) were cut and mounted on #200 square mesh grids and stained with lead citrate.

4.2.2. Morphometric analysis

Total volume of the DMV was measured in mm^3 using the serial Nissl sections through the nucleus. The caudal extent of the DMV was defined as the most caudal limit containing three or more motor neurons, which is situated in the caudal medulla. The rostral extent of the DMV was defined as the disappearance of clustered motor neurons nearing the pontomedullary junction. Individual section were examined at a final magnification of x265. The area of the DMV on every second section was measured in mm^2, using a digitizing tablet of an image analysis system (Bioquant System IV, R&M Biometrics, Nashville, TN). The number of sections analyzed per nucleus ranged from 43-50. The total volume of the DMV was calculated by the following equation:

$$V = \sum A \times T \times P,$$

where $\sum A$ is the sum of the area measurements, T is the thickness of the section, and P is the periodicity of the sample. In this case, the thickness of each section was 30 μm, with a periodicity of 2.

Twelve electron micrographs were randomly photographed across the DMV of each animal at a magnification of X4,800 using Kodak #4489 plate film. The negatives were developed in D-19 for 4 minutes and individually examined using a dissecting microscope at X6-16 for a final magnification of x28,800-76,800. Synaptic contacts were identified by the presence of a synaptic cleft between two parallel differentiated membranes, and the presence of three or more synaptic vesicles in close proximity to the presynaptic membrane. The N_V of synapses was determined as the number of contacts per mm^3 using the following formula:

$$N_V = N_A / (L + T),$$

where N_A is the number of synaptic contacts per unit area of electron micrograph, L is the mean length of individual contacts, and T is section thickness (Weibel, 1979; O'Kusky and Colonnier, 1982). The total number of synapses in the DMV of each animal was calculated by multiplying the N_V for that animal by the corresponding mean volume for that age.

Statistical significance of differences among the means at the various postnatal ages was determined by single factor analysis of variance. The statistical significance of differences between individual pairs of means was analyzed by the Student-Newman-Keuls method, with values of $P < 0.05$ considered statistically significant.

4.3. Results

The boundary of the DMV was clearly distinguishable at all ages by the high density of the medium sized motor neurons intensely stained for Nissl substance. The criteria for distinguishing the boundaries of the DMV during postnatal development in the rat are virtually identical to those in the mouse (for example, see Fig. 3.1). The caudal extent of the DMV was located in the caudal medulla, lateral to the central canal, dorsal to the hypoglossal nucleus, ventral to the nucleus of the tractus solitarius, and dorsomedial to the medullary reticular nucleus. The rostral extent of the DMV was defined as the disappearance of clustered motor neurons near the pontomedullary junction.

Postnatal changes in the total volume of the DMV are illustrated in Fig. 4.1.

The total volume of the nucleus increased gradually from approximately 0.0272 mm^3 at postnatal day 1 to 0.1241 mm^3 in the adult (457%, F= 141.724, df= 7, 56, P< 0.0001), with the most rapid increase being between postnatal day 1 and 5 (67%). Volume of the DMV continued to increase by 16% between postnatal days 30 and 120.

There was a significant increase in the N_V of synapses in the DMV (Fig. 4.2) from approximately 94 million synapses per mm^3 at postnatal day 1, to approximately 256 million synapses per mm^3 at postnatal day 30, followed by a significant decrease to approximately 179 million synapses per mm^3 in the adult (F= 34.534, df= 7,8, P< 0.001). These changes represent a 160% increase in the N_V of synapses from birth to day 30, followed by a decrease of 27% in the young adult. The initial increase and subsequent decrease in the N_V of synapses was due to parallel changes in the N_A of synapses (f= 37.044, df=7,8, P< 0.001) with no significant changes in the mean length of synaptic contacts (F= 0.683, df= 7,8, P> 0.05) during the course of the experiment. The mean contact length varied from 0.307 µm to 0.328 µm across the 8 ages studied (Fig. 4.3).

The total number of synapses in the DMV (Fig. 4.4) increased significantly from 2.57 million synapses at postnatal day 1, to 26.38 million synapses at day 30, followed by a significant decrease to 22.19 million synapses in the adult (F= 198.767, df= 7,8, P< 0.001). These changes represent a 928% increase in total synapse number from birth to day 30, with a 16% decrease in young adults.

4.4. Discussion

Volume of the DMV was found to increase by 457% during postnatal development in the rat. N_V and total number of synapses revealed a significant increase from birth to postnatal day 30, with a subsequent decrease to adult levels at postnatal day 120. The magnitude of this increase was greater for total number of synapses (928%), than for N_V (160%), due to the corresponding increase in volume. However, the magnitude of synapse elimination after postnatal day 30 was greater for N_V (27%) than for the total number of synapses (16%). Again, this was due to a gradual increase in the volume of the DMV continuing after day 30. The mean contact length for synapses in the DMV did not change consistently during postnatal development having a value of approximately 0.30 μm. Evidently, after initial synapse formation, there is no appreciable growth in the length of individual contacts during postnatal development. This finding has been shown by several other studies of synaptogenesis in the central nervous system (Cragg, 1975; Winfield, 1981; O'Kusky and Colonnier, 1982; O'Kusky et al, 1996; O'Kusky, 1998).

Although the results of the present study revealed the time course of synapse proliferation and elimination in the DMV during postnatal development, the parent neurons giving rise to the transient synaptic contacts cannot be identified in a morphometric analysis such as this. The major afferent projections to the DMV and dorsal vagal complex include the primary vagal afferents (Berthoud et al., 1990) and the descending suprabulbar projections to the DMV. These descending central nervous system projections include afferents from the paraventricular nucleus (Sofroniew and Schrell, 1981), dorsomedial and lateral hypothalamus (van

der Kooy et al., 1984), central nucleus of the amygdala (Higgins and Schwaber, 1983), bed nucleus of the stria terminalis (Holstege, 1987), and the insular and medial prefrontal cortex (van der Kooy et al., 1984). Synapse elimination from the developing DMV could involve a decrease in the complexity of the terminal arborizations of axons originating from neurons in these regions. On the other hand, afferent projections from uncharacteristic regions may contribute to the progressive phase of synaptogenesis in the DMV during fetal development, only to be completely eliminated during the regressive phase. In pathological situations, where these normally transient axons persist, their presence in the adult brain would be interpreted as an anomalous projection. Tract-tracing studies to demonstrate the origins of these transient projections to the DMV remain to be performed.

A previous study, which investigated postnatal changes in the N_V and total number of synapses in the hypoglossal nucleus during postnatal development (O'Kusky, 1998), was based on histological specimens from the same animals used in the present study. Postnatal development of the hypoglossal nucleus in these rats was characterized by a 414% increase in volume from birth to the young adult, which is comparable to the growth found in the DMV. The N_V and total number of hypoglossal synapses increased significantly from birth to peak values at postnatal day 20 (131% and 843%, respectively). The subsequent decrease in N_V was found to be 45% in the young adult, while the decrease in total number was found to be 30% (O'Kusky, 1998). Given the fact that the DMV lies immediately adjacent to the hypoglossal nucleus, it is quite remarkable that peak synaptic densities are achieved 10 days earlier in the hypoglossal nucleus than in the DMV. The

mechanisms by which extraneous synapses are eliminated in the brain are open to speculation. Growth factor hypotheses propose that sufficient uptake of a neuronal growth factor from the postsynaptic target site ensures survival of the synapse. Connectivity hypotheses argue that synapse elimination is selective and dependent on neuronal activity and functional validation (i.e. matching the activities of specific afferent and efferent axonal projections within a given nucleus). The synchronous elimination of synapses from adjacent but functionally dissimilar brainstem nuclei during development would tend to support growth factor hypotheses, assuming generalized distribution across the region. Sequential elimination of synapses from adjacent nuclei would tend to support connectivity hypotheses. The differential timing of synapse elimination in the DMV and the hypoglossal nucleus, would tend to support the connectivity hypotheses.

The hypoglossal nucleus controls tongue movement (Lowe, 1981). Primitive reflexes in neonatal rodents include suckling and rooting, which require sufficient maturation of tongue musculature and innervation to function at birth. The DMV, where 90% of its efferents innervate the stomach, and a minority of efferents innervate the heart and other viscera, might be expected to have a delayed maturation when compared to the hypoglossal nucleus. Rodents wean at approximately postnatal day 25. The change in gastrointestinal motility accompanying the ingestion of solid food, may require later consolidation and reorganization of axons innervating the gastrointestinal tract.

The differential time course of development in the DMV and the hypoglossal nucleus, on the other hand, could be explained by a differential expression of a

neuronal growth factor in the adjacent nuclei. One possibility could be that the DMV and the hypoglossal have requirements for different growth factors which function to control the progressive and regressive phases of synaptogenesis. No likely growth factor candidates have been identified to date. Another possibility is a differential time course in the expression of the same growth factor in the two adjacent nuclei. For example, IGF-I and its type I receptor have been shown to be diffusely distributed in the brainstem (Bondy, 1991; Bondy et al, 1992). This distribution has been described qualitatively using antibodies against IGF-I in mmunohistochemical studies and probes against mRNA by *in situ hyridization.* Although transient IGF-I gene expression has been qualitatively described to be variable among neighbouring nuclei, quantitative experiments relating concentration of IGF-I or rates of IGF-I gene expression to the N_V of synapses in various brainstem nuclei remain to be performed.

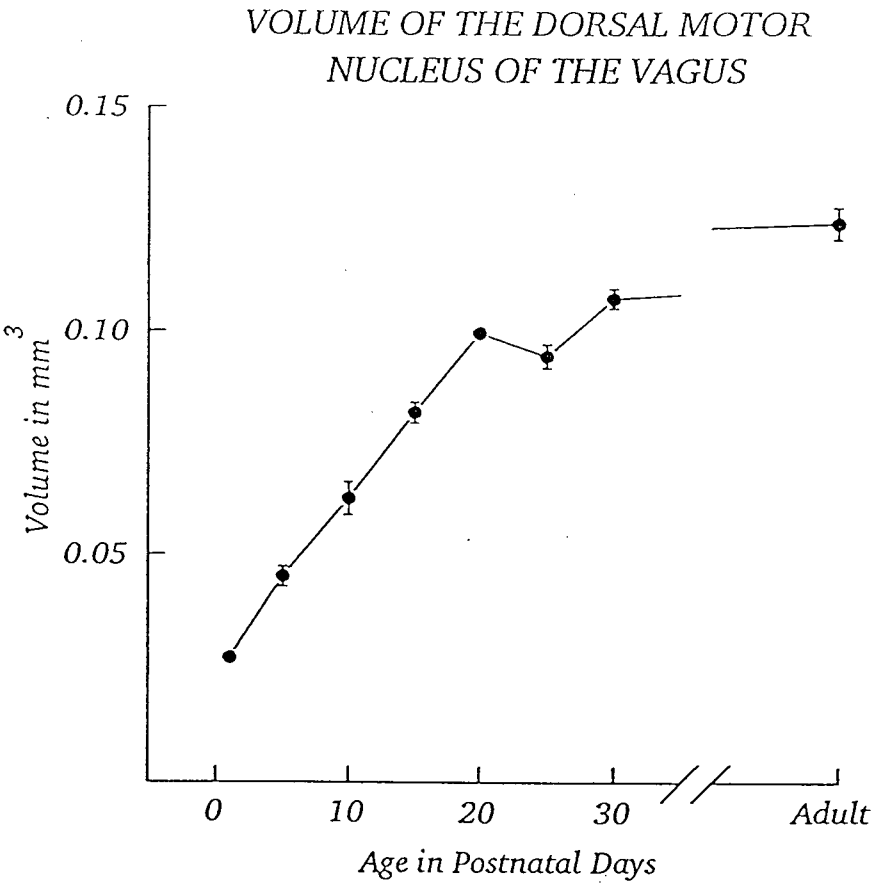

Figure 3.1. Postnatal changes in the total volume of the DMV in Sprague-Dawley rats. Values are given as the mean ± SEM for 8 nuclei at each age.

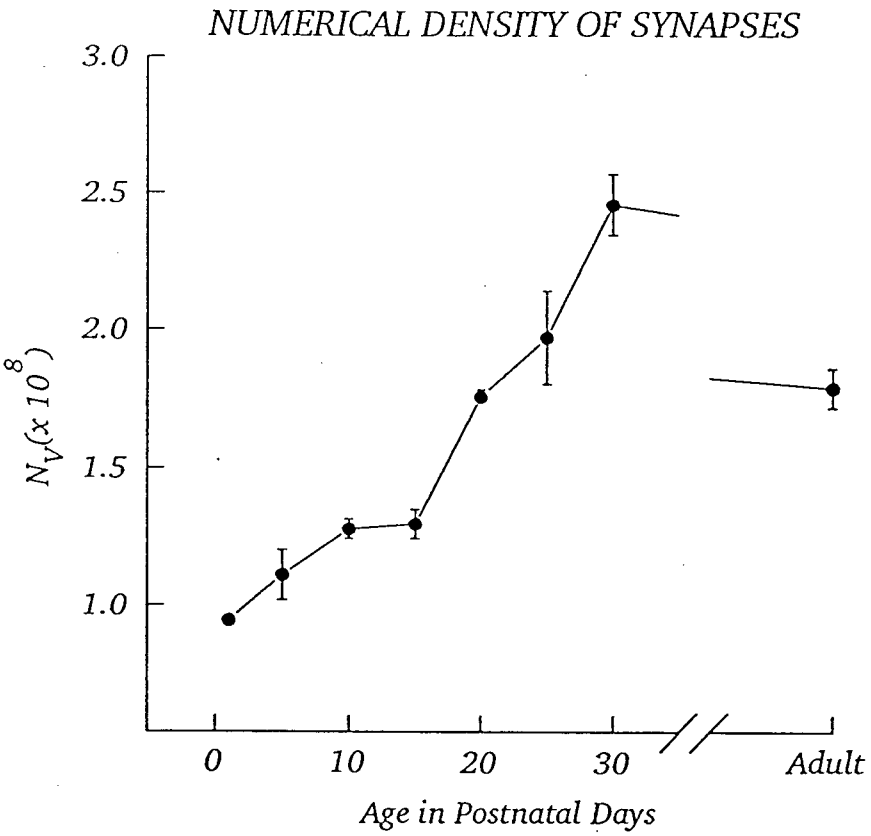

Figure 4.2. Postnatal changes in the N_V of synapses in the DMV of Sprague-Dawley rats. Values are given as the mean ± SEM for 2 animals at each age.

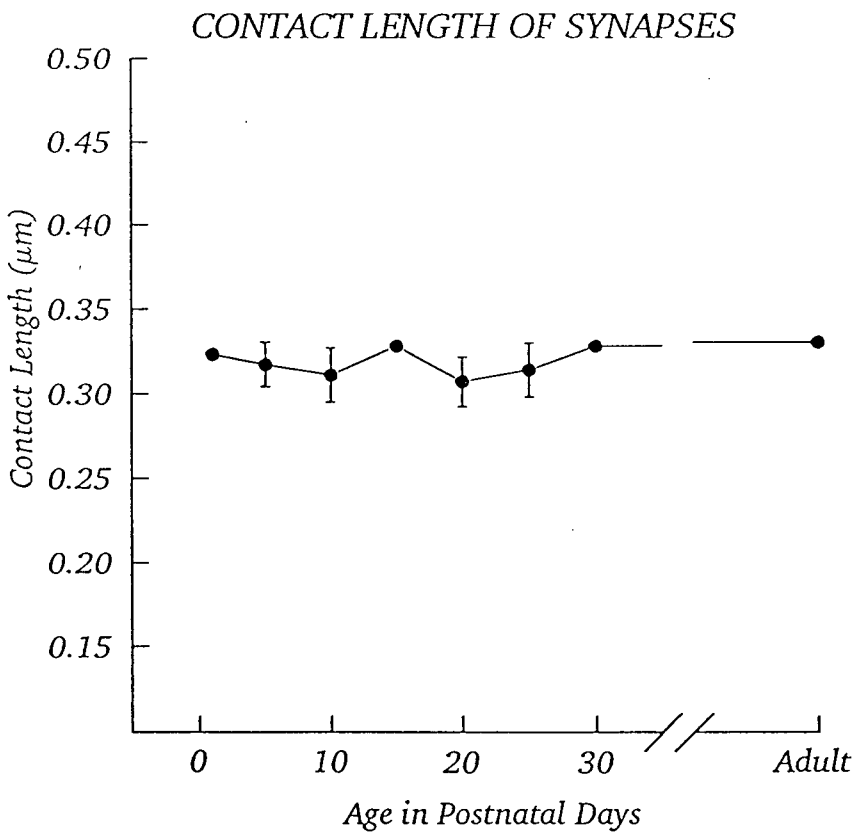

Figure 4.3. Postnatal changes in the mean contact length of synapses in the DMV of Sprague-Dawley rats. Values are given as the mean ± SEM for 2 animals at each age. Individual means were calculated from 100 randomly selected synapses per animal.

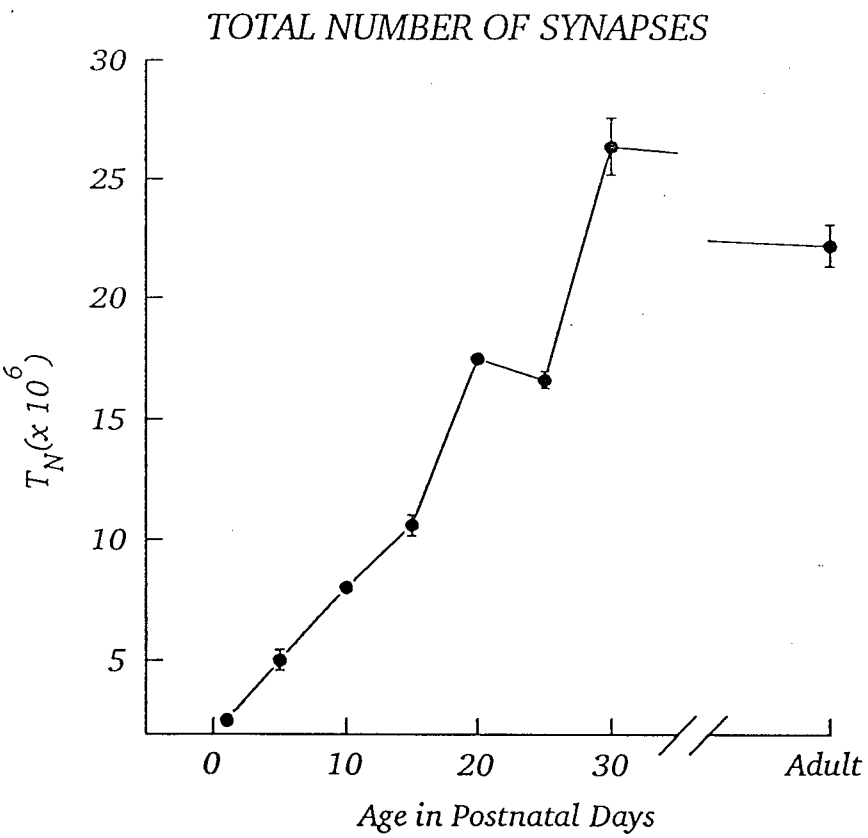

Figure 4.4. Postnatal changes in the total number (TN) of synapses in the DMV of Sprague-Dawley rats. Values are given as the mean ± SEM for 2 animals at each age.

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Chapter 5.

Summary and Significance

5.1. Morphometric analyses of the dorsal motor nucleus of the vagus in sudden infant death syndrome

The most pronounced finding in the present morphometric study of SIDS was the increased volume of the dorsal motor nucleus of the vagus, when compared to controls without neurological disease. The 33% increase was found to be due to an expansion in the area of the nucleus on individual sections in the transverse plane, and not in the length of the nucleus along the neuraxis. The number of neurons per unit volume (numerical density) was lower in SIDS than in controls, but because the volume was larger, the total number of neurons did not differ between the two groups. The mean neuronal profile area of motor neurons was found to be larger in SIDS than in controls, with very little overlap. This does not, however, account for the total volume increase of the DMV. Therefore, this lower neuronal packing density in SIDS reflects an increased volume of neuropil separating the neuronal cell bodies. The neuropil includes glial processes, dendrites, axons and synapses. In the present study, there did not appear to be any noticeable increase in the density of glia in the DMV.

Previous studies have found that there was an increase in the number of

dendritic spines on neurons of the DMV in SIDS victims (Takashima et al, 1994; Becker and Zhang, 1996). Dendritic spines are the main postsynaptic target sites for the majority of neurons in the primate brain (for review see Coss, 1985). Takashima et al. (1994) established the number of spines in the DMV to peak at 36-40 weeks of gestation during normal development. After birth, the regressive phase of synaptogenesis begins and the number of spines rapidly decreases (Takashima and Becker, 1986). This loss of dendritic spines is regarded as a normal developmental process, which represents the elimination of extraneous spines (Sotelo et al., 1975). Both Takashima et al. (1994) and Becker and Zhang (1996) suggest that the increase in dendritic spines in the DMV may be due to a failure to eliminate during the regressive phase of synaptogenesis. This persistence of spines has been noted in other respiratory and reticular nuclei of the medulla (Quattrochi et al., 1985; Takashima and Mito, 1985; Becker and Zhang, 1996). Our lab has demonstrated an increased number of synapses in the hypoglossal nucleus and in the central medullary reticular nucleus of infants that have died from SIDS (O'Kusky and Norman, 1995; O'Kusky and Norman, 1994). The increased volume of neuropil found in the DMV of SIDS cases in the present study, together with reports of the persistence of dendritic spines in the DMV (Takashima and Becker, 1985; Takashima et al., 1994; Becker and Zhang, 1996), strongly suggest an increase in the number of synaptic contacts in this nucleus. Future studies to quantify the N_V and total number of synapses in the DMV are planned, using the ethanolic-phosphotungstic acid method which selectively and reliably stains synaptic contacts in human autopsy material. Since the time course of the

progressive and regressive phases of synaptogenesis in the human brainstem has been extrapolated from studies of dendritic spine density on Golgi-stained material, studies of developmental changes in synapse number by electron microscopy throughout gestation and over long-term postnatal development need to be performed.

If the pathogenesis of SIDS involves the failure to eliminate normally extraneous synapses in the brainstem, then which specific axonal projections are being retained in susceptible infants? This persistent axonal circuitry would be potentially lethal in the infant, while it is presumably essential or at least benign in the fetus. One possible explanation involves the central nervous system control of fetal breathing movements. Rapid irregular contractions of the diaphragm and intercostal muscles are a natural feature of fetal development (Jansen and Chernick, 1983). The occurrence of fetal breathing movements appear to be required for the normal growth and development of the lungs (Alcorn et al., 1980; Liggins et al., 1981). Since fetal breathing movements are associated with increased oxygen consumption (Rurak and Grubner, 1983), their inhibition during periods of increased metabolic demand would be beneficial to the fetus. Hypoxia in the fetus produces almost complete inhibition of fetal breathing movements (Johnston, 1991), as opposed to the increased respiration seen in the adult.

Experimental studies in the unanesthetized fetal lamb *in utero* have demonstrated descending inhibitory pathways to the medulla, which are active during the apnea associated with fetal hypoxia (Dawes et al., 1983; Gluckman and Johnston, 1987; Johnston and Gluckman, 1989)Transecting the brainstem at the

level of the caudal mesencephalon and rostral pons resulted in continuous fetal breathing movements during episodes of hypoxia (Dawes et al., 1983). Focal lesions of the brainstem were employed to identify an area in the rostral pons, which was responsible for the inhibition of fetal breathing movements during hypoxia (Gluckman and Johnston, 1987; Johnston and Gluckman, 1989). These authors reported that the effective region included the lateral parabrachial nucleus and Kolliker-Fuse nucleus. In the newborn lamb, electrical stimulation in the ventrolateral rostral pons has been shown to inhibit breathing (Noble, 1991). Within this region of the pons, individual neurons exhibiting increased discharge frequency during hypoxia have been mapped in both the lateral and medial parabrachial nuclei (Noble, 1991).

Some authors have suggested that these inhibitory pontomedullary pathways may persist postnatally to produce the secondary fall in ventilation in the neonate during the biphasic response to hypoxia (Johnston, 1991; Noble, 1991). This response can be present for several weeks after birth depending on the species. The physiological mechanisms underlying the transitory effects of hypoxia, from an inhibition of breathing movements in the fetus and neonate to a stimulation of respiration in the infant and child, need to be studied. One possibility in the elimination of the axonal projections and synapses associated with the inhibitory pontomedullary pathways during the neonatal period. The normal elimination of dendritic spines from neurons in various nuclei of the medulla during late fetal and early postnatal development (Takashima and Becker, 1985) would tend to support this hypothesis. The results of the present study suggest that inhibitory

pontomedullary axonal projections to the DMV, the dorsal vagal complex, or the dorsal regions of the medulla may be retained in SIDS. This could have the effect of lowering the threshold to the onset of apnea, or raising the threshold to arousal from episodes of apnea that occur normally. Tract-tracing studies using the fluorescent carbocyanine dye DiI are needed to identify specific anomalous axonal projections that are being retained in SIDS.

The causal factors resulting in a failure to eliminate dendritic spines and synapses in SIDS are unknown. It is tempting to suggest that this increased connectivity may be the cause of the cardiorespiratory instability seen prior to death in susceptible infants. If it were possible to prevent experimentally the normal elimination of synapses from the brainstem during development in an animal model, respiratory and cardiac physiology could be monitored easily.

5.2. The role of insulin-like growth factor I in the postnatal development of the dorsal motor nucleus of the vagus and the hypoglossal nucleus

We asked whether or not an overexpression of IGF-I could be involved in the abnormalities seen in SIDS. The IGF-II/I transgenic mice did not die suddenly and unexpectedly during the course of the experiment, although subtle respiratory deficits cannot be ruled out entirely. Future studies are required to monitor cardiorespiratory physiology in these animals during postnatal development.

Although the IGF-II/I transgenic mice, which overexpress IGF-I during early postnatal development, have very similar morphological abnormalities in HN as do SIDS cases, elevated IGF-I produced rather different morphological abnormalities in the DMV of IGF-II/I transgenic mice than were observed in SIDS. Most notably, the 56% increase in the total number of neurons in transgenic mice was interpreted as most likely being due to an antiapoptotic effect of IGF-I on naturally occurring neuron death during early postnatal development. Proof of this awaits the application of the TUNEL method (TdT-mediated dUTP-biotin nick end labeling) to histological sections from the DMV at various developmental stages, combined with morphometric analyses to quantify the number of apoptotic neurons in transgenic and control mice. Since the time course of overexpression of IGF-I in these transgenic mice could not be manipulated, the fact that the brainstem abnormalities in transgenic mice did not match exactly those reported in SIDS may simply be a matter of timing. Elevated IGF-I in a slightly older mouse, after the period of neuron death but before the regressive phase of synaptogenesis, could conceivably produce the same developmental neuropathologies described in Chapter 2. The stereotaxic injection of small quantites of recombinant IGF-I into discrete brainstem nuclei could have the desired effect. For that matter, the efficacy of any neurotrophic factor (i.e. one which can be prepared or purchased commercially) to prevent synapse elimination could be tested in this model system. Prior to these studies, the time course of the progressive and regressive phases of synaptogenesis must be determined in individual brainstem nuclei.

5.3. Synapse elimination during normal development of the dorsal motor nucleus of the rat

The progressive phase of synaptogenesis in the DMV of rat occurs between birth and postnatal day 30. The regressive phase of synapse elimination occurs after day 30. This represents a very different schedule of synaptogenesis than that seen in the adjacent HN. This was an unanticipated finding, of interest to developmental neurobiologists concerned with the issue of various growth factor hypotheses versus assorted connectivity hypotheses. From the strictly pragmatic standpoint of one who is concerned with the pathogenesis of SIDS, these results dictate that the time course of synaptogenesis be investigated in most if not all of the cardiorepiratory nuclei in the medulla and pons. While this represents a substantial volume of work, the potential value of an animal model for screening growth factors that may be involved in the pathogenesis of SIDS and related cardiorespiratory disorders of development would justify the endeavor.

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