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Effects of Perinatal Undernutrition and Massage Stimulation upon the Ambiguus Nucleus in the Rat Prior to Weaning

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ABSTRACT

Undernutrition produces morphological brain alterations and cognitive deficiencies in children of underdeveloped countries. The central nervous system (CNS) alterations mainly interfere with the anatomical organization of areas undergoing a phase of intense postnatal cell proliferation, disrupting plastic processes like learning, memory, and phonation. In the rat pup, prenatal malnutrition interferes with the elaboration of ultrasonic vocalizations (USVs) by poorly understood mechanisms. The neuronal caudal portion of the nucleus ambiguus (Ambc) innervates the laryngeal intrinsic muscles to produce phonation, a basic USV communication system. During postnatal development, enhanced plasticity phenomena play a fundamental role in improving brain function. Thus, the massage stimulation (MS) may accelerate growth and induce neurogenesis in different areas of the brain. The current study analyzed the effects of a daily 10-min MS on the dendritic tree and perikarya measurements of Ambc multipolar motoneurons (Golgi-Cox) of perinatally underfed (U), control (C), control massage-stimulated (CMS), and underfed massage-stimulated (UMS) groups at postnatal days (PDs) 8, 12, and 15. The data indicated that the dendritic scores were reduced ($p < 0.05$) in both number and density at PD8 and PD15 in the U subjects and that MS increased the values of these parameters ($p < 0.05$). In addition, MS induced body weight gain in both U and CMS groups, and it enhanced the dendritic density in CMS subjects. These results show that MS during the pre-weaning period restores the plastic properties of the Ambc over the hypoplastic multipolar motoneuron after the alterations caused by perinatal undernutrition.

Keywords: Nucleus Ambiguus; Development; Massage Stimulation; Perinatal Undernutrition; Rat Pups

1. Introduction

In mammals, the mother-infant interaction plays a fundamental role in species preservation by eliciting plastic brain changes in the mother that produce maternal behavior triggering adaptive mechanisms in the newborn that promote survival in the nest environment [1-3]. In this context, rat pup's USV has been studied in a number of distress situations such as separation from the mother (frequency range from 30 to 50 Khz), exposure to cold, acute isolation, and pain (at 22 Khz) [4-7]. Furthermore, the characteristics of these ultrasonic calls are altered by prenatal malnutrition, presumably associated with damage of various CNS structures [8], particularly the brain stem areas associated with swallowing, breathing, and laryngeal motor innervations, which are necessary for phonation [9-11]. The neuroanatomical tracing studies provide a description of the central brainstem connections of the axons within the superior laryngeal nerve and recurrent laryngeal nerve, the latter with a special motor innervation to the intrinsic laryngeal muscles arising from the caudal portion of the Nucleus Ambiguus (Ambc) motoneurons [12-14]. These muscles and vocal fold morphology are modified in postnatally underfed rat pups [15].

Early nutritional deficiencies and postnatal environmental stimulation are some of the non-genetic factors that affect developing brain morphology. Undernutrition significantly interferes with brain growth by reducing neurogenesis, the number and density of dendrites, spinogenesis and the number of synaptic contacts in different brain areas [16-18], thereby producing an immature brain, with poor sensorial organization and affecting the transmission of ascending information patterns to the cerebral

cortex [19,20]. Food restriction during the perinatal period also alters the function of the Hypothalamus-Pituitary-Axis (HPA) causing inappropriate feedback mechanisms, since "fetal programming" disrupts both the short- and long-term adaptive responses to stress [21-23]. Moreover, perinatal undernutrition elicits long-term deficiencies in the maternal care of the progeny such as reduced nursing time, retrieval of pups, and body licking, and it increases non-maternal behaviors such as exaggerated self-grooming bouts that diminish the physical contacts with the young [24,25].

During the nursing period the mother-litter bonds are relevant for the development of the newborn rats who receive and provide direct sensorimotor cutaneous stimulation from littermates and from the mother's fur, maternal body licking, whisking movements, motor activity, and important vestibular activation during the huddling, and suckling [26-30]. In the last few years various handling routines enriched environments, and chronic tactile stimulation or body massage have been used with salutary effects on functional neuronal rehabilitation following several types of brain damage including that due to early nutritional deficiencies [25,30,31]. Thus, neonatal tactile stimulation increases the neurogenesis, and the number of dendrites and spines in the hippocampus, amygdala, and the cerebral cortex [32,33]. However, little is known about the effects of the chronic body massage and whether it can restore normal morphology after neuronal alterations associated with the perinatal undernutrition, particularly in the brainstem structures underlying the basic ultrasound vocalization in the rat.

Because underfed newborns interact poorly with their mother and littermates, and the maternal care of the pups is affected by reduced physical contacts, and ultrasound vocalizations, we hypothesize that neonatal body massage stimulation may overcome alterations of multipolar neurons in the Ambc due to perinatal undernutrition.

2. Material and Methods

2.1. Subjects

Animals were Wistar rats (250 - 300 g), descendants of a stock originally obtained from Harlan Sprague-Dawley, (IN, USA), and they were maintained in a temperature- and humidity-controlled room on a 12 hr/12 hr light/dark cycle, with water and food (Purina chow) ad libitum. For mating, two males were placed in a plastic cage containing four virgin females (200 - 250 g). Sperm-positive females were placed individually in plastic maternity cages ($50 \times 30 \times 20$ cm^3) with grill tops and wood shavings as nesting material one week before parturition. The day of birth was referred to as PD 0 and 24 hr later pups were weighed and sexed, and four females and four males from each litter were randomly distributed among dams in order to minimize genetic and nutritional differences that may influence the experimental results. The presence of the bilateral thoracic and abdominal line of nipples and the shorter anogenital distance in the females were used as criteria for sex recognition [34]. Animal care and protocols were approved by local Animal Committees and were consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Control Group (C)

The C animals consisted of 15 male pups obtained from eight litters normally nourished by well-fed mothers with free access to food and water. After birth, pups were fed and handled by interchanging a pair of normally lactating mothers every 12 hr as previously described [35]. To evaluate the effects of the nutritional paradigms on physical growth, body and brain weights of subjects with different experimental treatments were noted.

2.3. Underfeed Group (U)

The U subjects (n = 15) came from at least eight different litters. The normal chow diet requirement was calculated by measuring the food intake of a group of 6 pregnant control rats (200 - 250 g) every week during a 21-day period. The resulting average food intake for each week was the basal level used to calculate the food-intake percentage of the U females.Thus, dams were fed from gestational day 6 (G6) to G12 with 50% (7.8 g) of the normal diet (Purina chow), from G13 to G19 with 70% (10.9 g), and then with 100% (15.6 g) of the same diet until parturition to avoid resorption or cannibalism of pups. This protocol was chosen because neurogenesis in the brainstem Ambc portion and afferent connectivity occur primarily from G16 to G21 [36]. At birth, prenatally underfed newborns were nursed by two gestationally underfed dams, in one of which, the main galactophorous ducts had been tied subcutaneously [35]. To continue the neonatal underfeeding paradigm, these two lactating dams were interchanged every 12 hr between litters on PDs 1 - 15. This cross-fostering procedure ameliorates the effects on the pups of maternal sensory deprivation. No attempts were made to measure food intake in pregnant dams or newborn rats. Approximately 80% of the total underfed subjects included here were undernourished during the light phase of the cycle. For the control groups, after parturition the mother in each litter was kept on an ad libitum diet of Purina chow and water at a temperature of 24˚C ± 2˚C and with 12 hr of light per cycle (lights on at 8:00 hr).

2.4. Control Massage-Stimulated Group (CMS)

This group included a total of 15 subjects obtained from

eight, well-nourished litters, nursed by a pair of well-fed dams with free access to food and water; however, they also received a daily body MS for a 10 min period from PD 4 to PD 15, to evaluate the effects of MS as a reference for statistical comparisons. After 10 min of body MS, the pups were gently returned to their home cage.

2.5. Underfed Massage-Stimulated Group (UMS)

This group included a total of 15 animals obtained from eight different litters; they were experimentally treated like the U pups but also received daily experimental MS for a daily 10 min span from PD 4 to PD 15 under the same schedule and environmental conditions as the CMS animals and were then returned to their home cage. To evaluate the effects of the dietary treatment, MS, and age, body and brain weights of pups with different experimental treatments were obtained.

2.6. Early Massage-Stimulation

Early MS stimulation of pups was done by separating them from their mothers and immediately giving them for a daily 10-min (PD 4 to PD 15) gentle individual massage on different parts of the body; a progressive sequence was carried out twice and consisted of touching, holding, and rubbing the pups from the hip to the head by circular trajectories (approximately 6 times/10 sec at each area) and then in the opposite direction (1 min). Thereafter, the extremities were massaged with different frequencies by rotary hand movements and extending and flexing the arms against the body (1 min). The abdominal and hip regions were similarly stimulated by massaging with circular movements (1 min). After this, the head was laterally moved and stimulated by flexor and extensor movements (1 min). Finally, gentle body rotary movements were applied for a 1-min span. Thus, early MS stimulation continuously modified the intensity and frequency of tactile, proprioceptive receptors, and nerve endings underlying the neuronal codes of sensory information [26]. The MS sessions were carried out once a day between 9:00 and 10:00 h in a small plastic cage (30 × 23 × 15 cm^3) with 3 cm of wood shavings on the cage floor: the cage was covered with a woolen rag, and the experimental area was maintained at 27˚C under a red light of a 75 W lamp in a sound-proof room separated from the ambient noise of the main laboratory. At the end of the MS treatment, the pups were returned to their home cage, where they frequently received extra stimulation from the dam (anogenital and body licking, retrieving, and touching) [27]. Pups under all of the different experimental treatments were maintained with the mother in their habitat until the next MS session or the day of sacrifice for the histological procedure.

2.7. Histology

A total of 60 male rats were used for the two nutritional conditions, two MS conditions, and three ages (12 groups, 5 animals/group). Before sacrifice, subjects were weighed, deeply anesthetized with ether, and perfused transcardially first with saline and then with buffered 4% paraformaldehyde (JT Baker, Co), pH 7.4, at PDs 8, 12, and 15. Thereafter, subjects were decapitated; the brains were removed, weighed wet, cut into three coronal blocks, and immersed in a Golgi-Cox solution for impregnation. Three weeks later, the blocks were dehydrated and embedded in low-viscosity nitrocellulose. Subsequently, they were cut in coronal sections of 120 - 150 μm and mounted serially. The slides were coded to ensure blind evaluation with respect to age and dietary and MS treatment of subjects. During the neuronal image digitizing, the experimenter had access only to the code numbers and not to the ages and experimental conditions of the brain material. Identification and location of the Ambc were based on Paxinos and Watson's atlas [37]. Anterior-posterior coordinates for the localization of the Ambc corresponded to values ranging from Bregma 13.24 - 13.68 mm. For each group a total of 20 multipolar neurons, the most frequent type of neuron projecting to the laryngeal muscles were analyzed.

2.8. Morphometric Measurements

A total of 240 well-impregnated, multipolar neurons underlying the Ambc whose dendritic field was confined to one section were evaluated in each experimental condition, age group, and neuronal parameter (**Figure 1**). Dendritic arbor measurements were obtained by counting the number of 1st, 2nd, 3rd, 4th, and 5th dendritic orders. Dendritic branches leaving the cell body were defined as first order, while those which branched from the former were considered second order, and so on. The dendritic arborization was measured by placing the cell body and primary dendrites at the center of the first of a series of seven concentric rings (spaced at 40 μm intervals) and counting all dendritic intersections with larger individual rings [38]. Additionally, the cross sectional area and the perimeter of the Ambc neuron perikarya were measured. In all cases neuronal measurements were obtained at a magnification of 40 X using an image digitizing system (Perception Analysis System by Human-Computer Interface, Cambridge, UK). No attempt was made to correct for compression of the three-dimensional dendritic arbor to a two-dimensional sketch, since the relative differences between neurons remain constant when transformed from three to two dimensions [39]. Moreover, because the dendritic arbor is confined to the tissue section, no stereological method was used. Additionally, for the soma parameters the image analyzer did some calculations similar to

those previously described.

2.9. Statistics

Separate sets of statistical analyses were used to compare the score differences among ages and dietary conditions: 1) scores for body weight at day 1 of age before MS were compared by a one-way ANOVA, 2) score body weight were compared using a three-way ANOVA, 2 (nutritional conditions) X 2 (MS stimulations) X 4 (ages) or 3 (ages), for the wet brain weight were analyzed with a three-way ANOVA; 3) the role of undernutrition on the dendritic order and crossings of branches during development was compared by using a four-way ANOVA, 2 (nutritional conditions) X 3 (ages) X 2 (MS stimulations) X 5 (dendritic orders), or 7 (concentric rings) both as repeated measure. To detect cumulative effects (Total) of undernutrition on the two dendritic measurements, the effects of the diet on all dendritic arbour at various ages, and on the total number of dendritic orders or dendritic crossings, a two-way ANOVA was used, 2 (nutritional conditions) X 3 (ages). The statistical differences between experimental groups were compared using the Fisher LSD post hoc test. The threshold level for significance was set at $p \leq 0.05$.

3. Results

3.1. Body and Brain Weight Effects

The three-way ANOVA of body weight measurements indicated statistically significant reductions associated with the diet, $F(1,64) = 275.95$, $p < 0.0001$, age, $F(3,64) = 368.76$, $p < 0.004$ and MS, $F(1,64) = 60.40$, $p < 0.0001$; there was a significant interaction between the diet by age, $F(3,64) = 4.82$, $p < 0.004$, and MS by age, $F(3,64) = 11.06$, $p < 0.0001$. Post hoc comparisons indicated significantly lower body weight in the U group, at PDs 4, 8, 12, and 15; and at PDs 8, 12, and 15 in the UMS than the corresponding controls (**Table 1**). Additionally, brain weight comparisons indicated significantly reduced values for the diet, $F(1,48) = 60.47$, $p < 0.0001$, age, $F(3,64) = 368.76$, $p < 0.0001$, and MS, $F(1,64) = 60.40$, $p < 0.0001$, and there was a significant interaction between diet by age, $F(3,64) = 4.82$, $p < 0.004$. Post hoc comparisons indicated that brain wet weight values were lower in U, CMS, and UMS groups ($p < 0.05$) at PDs 8, 12, and 15 than controls (**Table 1**).

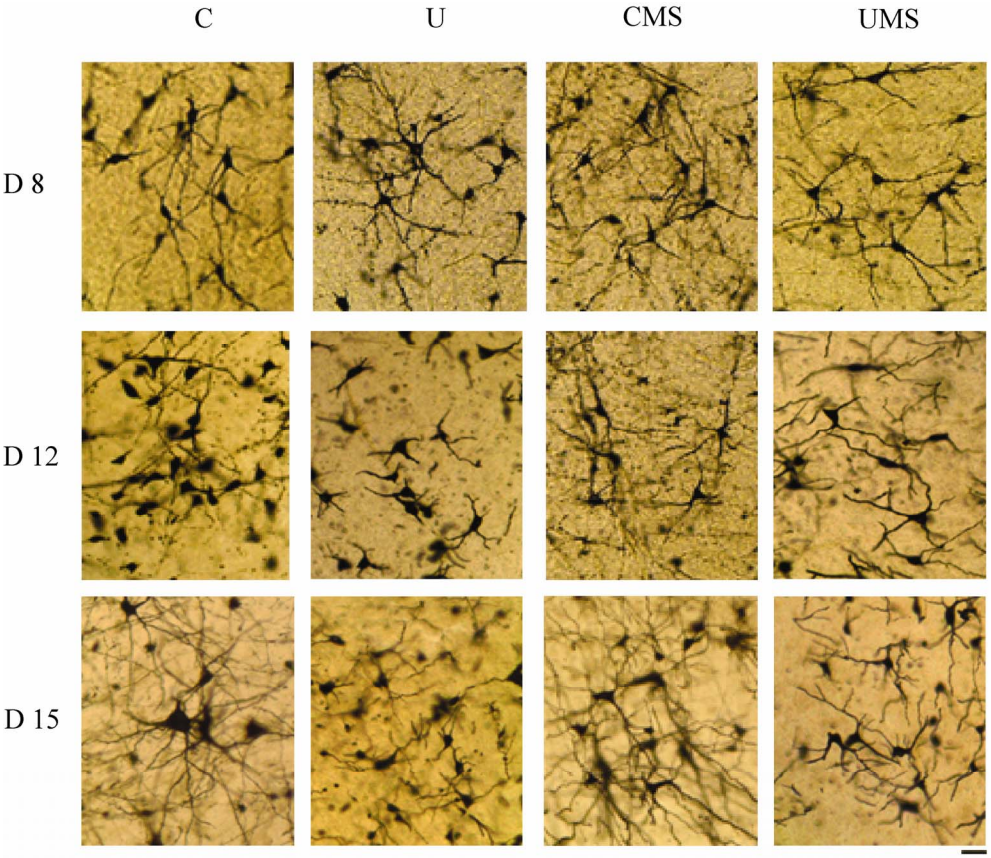

Figure 1. Representative photomicrographs showing coronal sections through the Ambc from C, U, CMS, and UMS at PD 8, PD 12, and PD 15. Note the increased dendritic arborization in the CMS, and the UMS compared to the C and the U rats. Bar = 100 µm.

3.2. Dendritic Tree Effects

The ANOVA comparisons of the dendritic orders between groups indicated significant reductions associated with age, $F(2,228) = 46.55$, $p < 0.0001$, the MS, $F(1,228) = 104.44$, $p < 0.0001$, and the number of branches, $F(5,1140) = 2591.35$, $p < 0.0001$. Furthermore, there were significant interactions between age by diet, $F(2,228) = 14.97$, $p < 0.0001$, and MS by age, $F(2,228) = 26.17$, $p < 0.0001$.

Post hoc comparisons showed that multipolar neurons exhibited reductions ($p < 0.05$) in the 3rd dendritic order of U vs. the C at the three ages studied. Furthermore, significant increases in the number of branches ($p < 0.05$) in the UMS vs. the U in the 3rd and 4th orders at PD 8, PD 12, and PD 15 were observed (**Figure 2(a)**). As well as significant interactions between MS by age by diet and by dendritic orders, $F(10,1140) = 10.03$, $p < 0.0001$. The cumulative effects of number of dendritic and density branches along the ages, and MS condition (Total) showed significant reductions ($p < 0.05$) in the total number of dendrites in the U vs. the C at PD 8 and PD 15, although there were significant increases ($p < 0.05$) in the CMS vs. the C pups at PD 12 and PD 15. The findings indicate that from the 3rd dendritic order onwards the dendritic orders of the UMS and the CMS groups increased consistently at PD 8 and PD 12 compared with the C group, with no effects at PD 15, while the CMS values remained elevated only with respect to the C group. Furthermore, among the U subjects most of the values for the 3rd to the 5th dendritic orders were reduced when compared to the C animals (**Figure 2(a)**).

The density of the dendritic branches of Ambc neurons, measured as the number of crossings of dendrites per circle showed significant reductions associated with diet, $F(1,228) = 18.54$, $p < 0.0001$, age, $F(2,228) = 72.77$, $p < 0.0001$, the MS factor, $F(1,228) = 166.94$, $p < 0.0001$, and the number of dendritic crossings, $F(6,1368) = 1226.55$, $p < 0.0001$. Moreover, there was a significant interaction between the age by diet, $F(2,228) = 47.18$, $p < 0.0001$.

Table 1. Mean values ± SEM of body and brain weight (g) in C, U, CMS, and UMS rats during development.

Pup's body weight at day 1 before MS				
C (n = 60)	**U (n = 60)**	**df**	**F**	**p <**
7.496 ± 0.066	6.656 ± 0.088*	1,118	57.15	0.0001

Age (days)	Groups			
	C (n = 5)	**U (n = 5)**	**CMS (n = 5)**	**UMS (n = 5)**
4	9.666 ± 0.178	6.708 ± 0.388	9.640 ± 0.293▾	7.996 ± 0.199
8	15.004 ± 1.214	7.546 ± 0.217	17. 094 ± 0.291▾	10.514 ± 0.666♦
12	20.854 ± 0.618	13.262 ± 0.597	24.088 ± 0.364▾	18.272 ± 1.650♦
15	25.082 ± 0.557	18.210 ± 0.937	28.640 ± 0.467▾	21.594 ± 0.542♦
Total	17.651 ± 1.381	11.431 ± 1.102	19.865 ± 1.658	14.594 ± 1.341♦
	Brain weight			
8	0.844 ± 0.017	0.600 ± 0.001*	0.960 ± 0.024	0.666 ± 0.023♦
12	1.260 ± 0.018	0.960 ± 0.090*	1.266 ± 0.022▾	1.052 ± 0.031♦
15	1.404 ± 0.023	1.151 ± 0.021*	1.380 ± 0.037▾	1.238 ± 0.009♦
Total	1.169 ± 0.064	0.903 ± 0.061*	1.202 ± 0.049▾	0.985 ± 0.064♦

Factors	Body weight			Brain weight		
	df	F	p <	df	F	p <
A) Diet	1,64	275.956	0.0001	1,48	358.20	0.0001
B) MS	1,64	60.407	0.0001	1,48	20.13	0.0001
C) Age	3,64	368.764	0.0001	2,48	597.13	0.0001
AxB	1,64	1.880	NS	1,48	3.70	NS
AxC	3,64	11.060	0.0001	2,48	3.01	NS
BxC	3,64	4.824	0.004	2,48	1.92	NS
AxBxC	3,64	0.363	NS	2,48	3.85	0.028

*C vs. U, ▾C vs. CMS, ♦U vs. UMS, Post hoc statistical differences between groups, $p < 0.05$. NS = Non significant values.

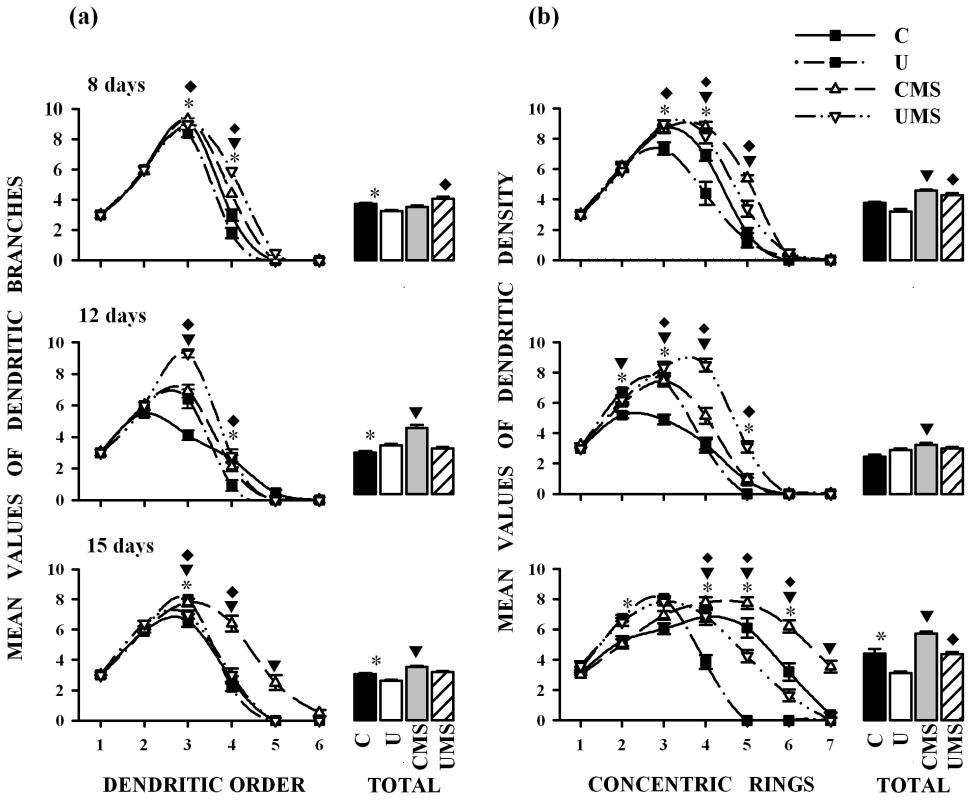

Figure 2. Mean values ± SEM for (a) dendritic orders and (b) dendritic crossings of multipolar Ambc neurons (n = 20/age group) of different experimental groups at different ages. Note, the reduced dendritic measurements at the distal portions of the arbor, in the U vs. the C rats as well as, the increased values in the CMS and UMS groups vs. the C and U groups. *C vs. U, ▼C vs. CMS, ♦U vs. UMS. The cumulative effects of undernutrition (see text) indicated significant reductions ($p < 0.05$).

Post hoc comparisons along the 7 circles indicated significantly fewer ($p < 0.05$) of crossings of U with the 3rd and 4th circles at PD 8; with the 2nd, 3rd, and 5th circles at PD 12; and with the 2nd, 3rd, 4th, 5th, and 6th circles at PD 15 when compared with the C subjects. Additionally, there were significant increases ($p < 0.05$) in the number of intersections in the CMS vs. the C group with the 4th and 5th circles at PD 8; with the 2nd, 3rd, and 4th circles at PD 12; and with the circles 3rd, 4th, 5th, 6th and 7th at PD 15. When the UMS group vs. U groups were compared a significant increases ($p < 0.05$) in the number of crossings with the 3rd, 4th, and 5th at days PD 8 and PD 12 and with the 4th, 5th, and 6th circles at PD 15 were observed (**Figure 2(b)**). The ANOVA also indicated significant interactions between MS by age by diet by the number of dendritic crossings, $F(12,1368) = 7.92$, $p < 0.0001$. The cumulative effects of undernutrition on the density of branches with increasing age (Total) indicated significantly reduced values ($p < 0.05$) in U vs. the C only at PD 15. Additionally, there were significantly more ($p < 0.05$) dendritic crossings in the CMS at PDs 8, 12, and 15 days, and in the UMS at 8, and 15 days of age compared to the C and U groups respectively. The dendritic density showed a similar increase for the 3rd to the 7th dendritic crossings as for the dendritic orders in the CMS compared to the C values at 8, 12, and 15 days and the UMS vs. U respectively, with reduced values in the U dendritic crossings on most of the days studied (**Figure 2(b)**).

3.3. Perikarya Effects

The ANOVA comparisons of the perikarya cross sectional area scores showed significant effects associated with age, $F(2,228) = 8.97$, $p < 000$, and the MS, $F(1,228) = 28.13$, $p < 0.0001$. Moreover, there were significant interactions between MS by age, $F(2,228) = 9.12$, $p < 0.0001$, MS by diet, $F(1,228) = 11.57$, $p < 0.0007$, and the age by diet, $F(2,228) = 6.03$, $p < 0.002$.

Post hoc comparisons indicated significant area reductions ($p < 0.05$) in the U vs. C animals at PD 8 and PD 12 (**Figure 3(a)**). Furthermore, significant area increases ($p < 0.05$) in the CMS vs. the C group only at PD 15; and there were significant increases ($p < 0.05$) in the cross sectional area values of UMS vs. U pups at 8, 12, and 15 days (**Figure 3(a)**). The cumulative effects of undernutrition on the cross sectional area values of the experimental groups with ages (Total) yielded a significant reduction ($p < 0.05$) in the U vs. the C group, and signi-

ficant increases ($p < 0.05$) in the scores of CMS vs. C, and the UMS vs. U subjects (**Figure 3(a)**).

Statistical comparisons of the soma perimeter values showed significant effects associated with diet, $F(1,228) = 14.76$, $p < 0.0001$, age, $F(2,228) = 4.31$, $p < 0.01$, and MS, $F(1,228) = 20.93$, $p < 0.0001$. Furthermore, a significant interaction between age and MS, $F(2,228) = 7.15$, $p < 0.0009$, was observed. Post hoc comparisons indicated significant reductions in the soma perimeter of the U vs. the C at PD 8; whereas significant increases ($p < 0.05$) in the values of the CMS vs. the C subjects at PD 15 were observed. Additionally, the values of the UMS vs. the U group were significantly increased ($p < 0.05$) at PD 8 and PD 15. The cumulative effects of undernutrition on the soma perimeter of the experimental groups with age (Total) showed significant reductions in the values of the U vs. the C group. Moreover, the soma perimeter values of the CMS vs. the C, and, the UMS vs. the U groups were significantly increased ($p < 0.05$), (**Figure 3(b)**).

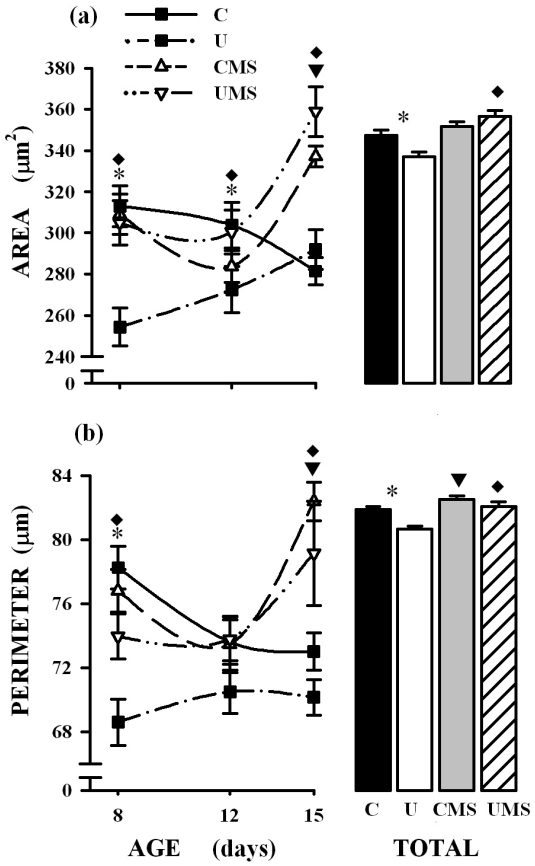

Figure 3. Mean values ± SEM of cross sectional area (a) and the perikarya perimeter (b) of multipolar Ambc neurons of C, U, CMS, and UMS groups at different developmental ages. Note, the reduced values of the U vs. the C subjects, and the increased values for the same parameters in the CMS and UMS groups. *C vs. U, ▼C vs. CMS, ♦U vs. UMS ($p < 0.05$).

4. Discussion

The present results indicated that, throughout the study, body and brain weights were consistently reduced in the U compared to C animals. Furthermore, the MS increased the body and brain weight at PD 15, and the results were also in line with previous studies showing that pre- and neonatal undernutrition in the rat delays physical and sensory development, ear- and eye-opening, and huddling [15,30,40-42]. This calls attention to the increased effects of MS on the body weight of CMS subjects compared with the other experimental groups, and the rehabilitation of MS effects of food deprivation in the UMS group. These effects could be related to the release of growth factors such as the epidermal growth factor (EGF) and growth hormone (GH) associated with tactile stimulation and MS that may accelerate body and brain weight gain. Giving support to this possibility is the fact that the EGF administration to the rat prior to weaning promotes incisors budding, eye-opening, and an increase in the ornithine decarboxylase (ODC) enzyme that stimulates protein synthesis in the CNS and peripheral visceral organs [32,43-47].

The current findings indicated that both pre- and neonatal food restriction resulted in a consistently smaller number of dendrites in the U subjects from the medial through the distal portions of the dendritic tree compared to the C animals. These effects were associated with a reduction in the dendritic density on the same portions of the tree as revealed by the reduced number of dendritic crossing. By contrast, increases in these same values and in the same portions of the tree were observed in the CMS and UMS groups, showing that the MS stimulation promotes the number and density of the dendritic orders thereby potentiating the integration of complex afferent nerve impulses to induce plastic changes in the discharge of Ambc motoneurons. The reverse effects may be true in the case of the U subjects, because the impoverished neuronal Ambc dendritic substrate, impairs integration of the spatiotemporal patterns of coding information needed to activate the laryngeal smooth muscles. These results are also supported by proposals that dendritic prolongations are involved in post-transcriptional processes through local protein synthesis at the level of the growth cone, and dendrites by the interaction between local proteins and neuronal microtubes [48,49].

Furthermore, it is possible undernutrition increases glucocorticoid levels which may interfere with the ODC enzyme activity, and the reduction in the BDNF content by undernutrition interferes the local protein synthesis causing neuronal plastic deficits [17,18,49,50].

Other points of interest concern the role of the MS and its interaction with perinatal undernutrition in the number of dendritic branches, theirs density and the effects on

cross sectional area and soma perimeter of Ambc multipolar neurons. The reduced MS effects in the U vs. the C is consistent with the fact that in neonatally underfeed dams impoverish their newborn by withholding the relevant tactile, retrieval and anogenital licking activity normally provided by the dam and necessary for pup survival and development [1,51-53]. Furthermore, the increased MS effects in the CMS, and UMS vs. the C subjects on the total dendritic arbor, and less consistent effects on the perikarya are supported by data using chronically repeated physical training or environmental enrichment that results in functional cognitive recovery, increased neurogenesis, and synaptogenesis in the CA1 subfield, cerebral cortex and hypothalamus [17,31,54]. Additionally, the fact that the MS promotes development in multipolar Ambc neurons of CMS and UMS subjects indicates that the neurons are independent of the nutritional condition, and may be the target of a common specific mechanism for protective synaptic plasticity and rehabilitation. These effects of MS could be related to the relationship of neuronal synaptogenesis with several growth factors, such as the brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and GH, that promote the recovery from neuronal deficits elicited by perinatal undernutrition, brain ischemic lesions, and movement restriction among others [3,31,32,55-58].

The salutary effects of the MS treatment used here may stimulate the release of several nerve growth factors that modify the plastic properties of multipolar Ambc neurons dendrites by various cellular and sub-cellular mechanisms resulting in increased dendritic complexity and synaptogenesis [49,59]. The MS paradigm is a potential therapeutic strategy to ameliorate early noxious effects associated with a number of perinatal risk factors that interfere with the plastic cognitive capabilities of the brain in adulthood. However, further studies are required to identify the advantages and limitations of this rehabilitation paradigm at different ages, experimental conditions, and time windows of brain development.

Finally, our data indicate that significant dendritic tree and perikarya hypoplasia of multipolar Ambc neurons was associated with perinatal undernutrition during the nursing period. Furthermore, daily 10-min MS stimulation from days 4 to 15 of age results in significant increases of the above-mentioned neuronal parameters in the CMS and UMS subjects compared to the C subjects. These findings may be related to cellular and subcellular neuronal mechanisms involved in neuronal dendritic tree and perikarya development possibly mediated by the release of growth factors associated with the neonatal MS paradigm. The current MS paradigm could be used for the rehabilitation from perinatal brain damage that if untreated may emerge at later ages as deficiencies in complex plasticity phenomena.

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