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DEVELOPMENT OF PREFRONTAL LAYER III PYRAMIDAL NEURONS IN INFANTS WITH DOWN SYNDROME

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Abstract

We quantitatively analyzed the dendritic and dendritic spine development on basal and oblique dendrites of large layer IIIc pyramidal neurons of the prospective prefrontal area 9 in the brains of three infants with Down syndrome (DS) and five age-matched-controls over the period from 32 weeks postconception to the 7th postnatal month. By using Neurolucida 3.1 software on rapid Golgi impregnated slices, 9-10 neurons were three-dimensionally reconstructed. There were no significant differences in the pattern of the dendritic and spine development between the basal and apical oblique dendrites. The DS subjects did not depart significantly from the developmental curve of the control subjects. Our data showed that large and significant segment outgrowth, in parallel with dendritic elongation occurred during a limited period of time, between 36 weeks postconception and the first postnatal month. Dendritic spines appeared at the time of birth and their density continued to increase up to the age of 7 months. During the first postnatal month long thin spines and filopodia-like protrusions predominated, but the spines later changed their morphology to a more mature form. No differences in the spine morphology were qualitatively observed between the DS infants and the age matched controls. This data suggests that intensive formation of cortical circuitry occurs on large layer IIIc pyramidal neurons during perinatal period and is not disturbed in DS infants. Consequently, this could be a biological potential to mitigate psychomotor impairment in DS patient.

Keywords

• Down syndrome • Association cortex • Layer IIIc pyramidal neurons • Rapid Golgi method • Quantitative analysis

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1. Introduction

Down syndrome is the most frequent form of mental retardation caused by genetic factors [1,2]. In spite of many neuropathological studies on Down syndrome [for review see 3,4], a common neurobiological basis to all these children is still elusive [5,6]. Children usually have reduced frontal lobes and prominently narrow superior temporal gyri [3,7-9]. The neocortical laminar formation is irregular, brain growth and the myelination of the cortical fibers (especially associative and commissural fibers from the frontal and temporal lobe) are delayed. There is a reduction of dendritic length and spine dysgenesis on the neocortical neurons [8,10,11]. Additionally, the neurons of Down syndrome patients were found to exhibit decreased spine density in a number of brain regions [12-15]. Therefore, it is supposed that cognitive dysfunction and speech abnormalities in DS may be caused by the disordered development of cortical circuitries [8,16]. Furthermore, the time course of neuropathological changes in Down syndrome displays regional patterns comparable with those observed in ageing and Alzheimer disease [17]. These neuropathological changes are consistently present in the association areas of the cerebral cortex, with associative-commissural layer III pyramidal neurons being a major vulnerable cell population [3,17]. It has been also suggested that the cortical changes in the brains of children with DS are secondary to maturation delay, retardation of growth, and cortical dysgenesis [8,18,19]. Thus, it seems that perinatal development of layer IIIc pyramidal neurons deserves close scrutiny, as these neurons are the key cellular element in the input-output organization of the corticocortical connectivity [20,21]. However, developmental studies of pyramidal neurons in DS are suprisingly scarce, and exclusively focused on the development of dendrites and the dendritic spines of the pyramidal neurons in the primary visual and primary sensori-motor cortex [12-14, 22-27].

During the past decade, we have collected an extensive set of data on normal prenatal and postnatal development of neurons and connectivity of the human frontal association cortex [20,28-30], with special attention to the development of the large associative layer IIIc pyramidal neurons [20,21,31-33]. This data offers a background for the analysis of the effects of developmental brain disorder on a selective neuronal population in a defined cortical area, in this case key cellular elements in the corticocortical circuitry [20,34-38].

In this context, we have studied the growth of the dendritic tree as well as the changes in spine density during the late fetal and early postnatal stage in infants with DS and in the

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children without any brain pathology or genetic malformation. The most rapid dendritic growth occurs on the associative layer IIIc neurons during this period, and their final bifurcation complexity is also reached then [32,33]. Our hypothesis was that the dendritic and spine development were not disturbed in DS infants during the analyzed period, implicating that the cellular and circuitry elements important for processing higher cognitive functions are well preserved during the early postnatal life. Preliminary pilot data of basal dendrite tree development in two DS cases have been previously presented [40].

2. Experimental Procedures

2.1 Brain tissue

We analyzed the postmortem brains of 8 subjects (3 subjects with Down syndrome: aged 36 weeks postconception, 2.5 and 4.5 postnatal months; and 5 control subjects, aged 32 and 40 weeks postconception, and 1, 2.5 and 7 postnatal months). All specimens were part of the extensive Zagreb Neuroembryological Collection and were collected and analyzed with approval of the Institutional Ethical Committee of the School of Medicine in Zagreb [41].

All of the analyzed subjects died of non-neurological causes and without a preagonal state, so that the postmortal interval actually represents the time of neuron death. In addition, no signs of brain pathology were noted in these specimens (Table 1)

2.2 Golgi rapid method

The tissue was prepared by the rapid Golgi method [21,42-44]. In brief, small blocks of tissue (1cm^3) were taken from the prospective area 9 of the superior frontal gyrus [21,43,44], then immediately stored in Golgi-Scheibel solution (0.3% osmium tetroxide and 3% potassium dichromate) and kept in the dark for 7 days [42]. The dichromate solution was then replaced with 1% silver nitrate solution for another 3 days. Subsequently, the tissue was dehydrated, rapidly embedded in 8% celloidin (nitrocellulose dissolved in ether-alcohol) and cut into 150-200 micrometers thick coronal sections.

Table 1. Specification of tissue sample.

Age	Case number	Sex	PMD (h)	Cause of death	N/T
32 pcw	cfp 304 - C	F	9	b.o.	10/160
36 pcw	cfp 240 - DS	F	7	Rh immunization	9/160
newborn	cd 96 - C	F	3	b.o.	10/140
1 pm	cd 147 - C	M	4	SIDS	10/160
2.5 pm	cd 146 - DS	M	4	Pneumonia	9/180
2.5 pm	cd 105 - C	M	6	Pneumonia	10/140
4.5 pm	cd 170 - DS	F	6	Insuf. cordis	8/180
7 pm	cd 123 - C	M	5	SIDS	10/165

Abbreviations: PMD, postmortem delay (in hours); N, number of neurons quantitatively studied; T, section thickness, pcw, postconception weeks; m. postnatal months; b.o., no signs of neuropathological changes, normal neurological status; F, female; M, male, C, control; DS, Down syndrome; SIDS, sudden infant death syndrome.

2.3 Quantitative analysis

From each specimen, 9-10 fully impregnated layer IIIc pyramidal neurons were selected for a three-dimensional dendritic reconstruction. The layer IIIc was defined as a 150 mm thick belt populated with pyramidal cells upon the unimpregnated layer IV [32]. The neurons selected for the analysis had completely impregnated basal dendritic processes which were not obscured by heavy clusters of dendrites or stain precipitations, and their soma were situated near the center of the thickness of the section in order to reduce the number of cut segments at the surface of the section. The soma size of analyzed cells should have been close to the size of largest layer V pyramidal neurons.

Quantitative analysis was performed using an Olympus Bx50 microscope with a 3D-motorized stage and attached Hitachi 3CCD camera, using 60x air objective (the software corrected the z-depth values by air refraction factor/1,515). The 3D-reconstruction and morphometric analysis were performed by means of Neurolucida 3.1 software (MicroBrightField, Williston, VT, U.S.A.). All pyramidal neurons were measured in the same sequence [45]; first the cell body was drawn in the 2-dimensional plane, and then the 3-dimensional dendritic reconstruction was performed. Parallel with the dendritic reconstruction, the position of dendritic spines was indicated.

The following parameters of both the basilar and apical oblique dendrites were separately analyzed: 1) the number of primary dendrites, 2) the total number of segments, 3) total dendritic length, 4) mean length of the individual intermediate and terminal segments [31,32,40,44]. The intermediate segments are segments between the dendritic origin and the bifurcation point, or between two bifurcation points [32]. Terminal segments are segments which do not bifurcate. Some segments ran into the precipitation, or ended on the section borders. These segments were defined as incomplete, and were excluded from the analysis of individual segment length and spine density on the terminal segments. Basal

dendrites are dendrites arising from the base of the cell body, and oblique dendrites are the side branches originating from the proximal part of the main apical dendritic tree.

The density of the dendritic spines was presented as a mean spine number per one micron dendritic length. Data were shown for the whole dendritic tree, as for the intermediate and terminal segments separately. All protrusions corresponding to the definition of dendritic spines according to Harris KM [46], were counted, including the hair like processes [47]. All data were separately presented for the basal and oblique dendritic tree.

2.4 Statistics

We applied the SPSS package for statistical analysis. The dendritic parameters and the spine number were tested separately with one-way analysis of variance with parametric and nonparametric analysis with age as a main effect. In the statistical analysis every subject represents a separate age. The a posteriori Student-Newman-Keuls test for multiple comparisons was applied to determine which subjects were significantly different. A P level lower than 0.05 was considered to be significant.

3. Results

3.1 Qualitative analysis

At 32 postconception weeks (pcw) the largest pyramidal neurons in the deep part of the layer III were very immature (Figure 1). Their body size were obviously smaller than the size of the large layer V pyramidal neurons, and the number of dendrites, as well as the level of their bifurcation was very low. From the top of the cell body, relatively thick apical dendrite ran towards the pial surface, bifurcating terminally on few thinner branches in layers II and I. The basal and the oblique dendrites were very thin, and mostly did not bifurcate. Their number was lower than in the older analyzed subjects. On some neurons the first dendritic spines could be observed on apical dendrites, but on basal and oblique branches they could not be found.

At the age of birth, the size of the cell body and dendritic thickness increased, as well as the number of bifurcations and the length of the segments (Figure 1). The oblique dendrites originated mostly in the first 200 mm of apical dendrite, almost under the right angle and take course parallel with the pial surface. The basal dendrites originated from the basal part of the cell body, and ran in all directions (*i.e.* from parallel with the pial surface to vertical into the layer IV). On all dendrites analyzed, dendritic spines could be observed, but their density was still very low. Some of them had typical hair-like morphology without terminal bulbs (filopodia-spines), and the remaining spines had long stalks with small bulbs.

At the first postnatal month, a further increase in the number of bifurcations (segments) and in the length of segments could be found (Figure 1). The size of the cell body, dendritic thickness and spine density increased. The morphology of spines did not change and hair-like spines predominated (Figure 2A). After this period no further increase in the number of segments, nor further elongation of dendrites could be observed. The apical dendrites became more frequently cut at the section border, and sometimes they could not be followed for longer distances.

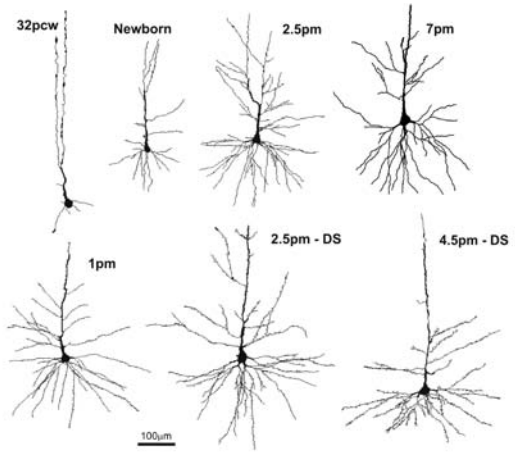

Figure 1. Three-dimensional reconstruction of layer IIIc pyramidal neurons. The basal and apical dendritic trees of layer III pyramidal neurons were reconstructed using Neurolucida software. Representative examples of reconstructed neurons at the following ages; controls: 32 pcw, newborn, 1 pm, 2.5 pm, 7 pm; and DS subjects: 2.5 pm, and 4.5 pm. Note that there are qualitatively no obvious changes which could indicate a disruption in the dendritic development in DS cases compared to the close age-matched control subjects. All layer IIIc pyramidal neurons are represented at the same magnification. Scale bar = 100 µm. Abbreviations: pcw, postconception weeks; pm. postnatal months; DS, Down syndrome.

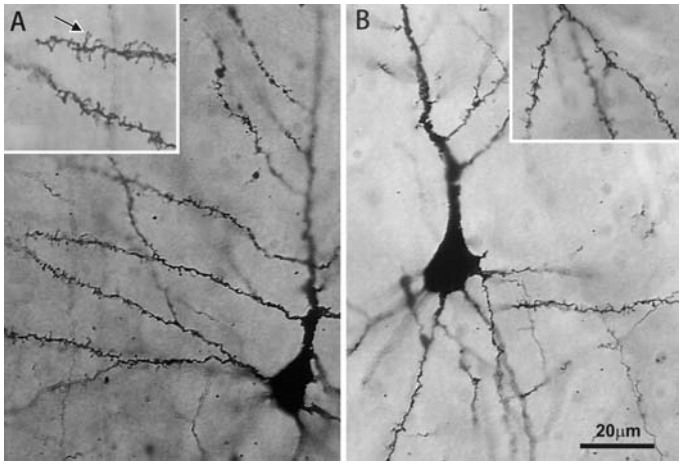

Figure 2. Golgi impregnated pyramidal neurons in the prefrontal cortex. Photomicrographs of representative rapid Golgi impregnated pyramidal neurons in control subject aged 1 postnatal month (A) and DS subject aged 2.5 postnatal months (B). Higher magnifications of selected dendritic segments are presented in upper left (A) and upper right corner (B). Arrow indicates hair-like spines. Scale bar = 20µm.

The basal dendritic tree achieved their typical orientation in the 2nd postnatal month, so that the majority of dendrites ran vertically into layer IV (Figure 1). Up to the 7th postnatal month, the size of the soma, the thickness of the dendrites and spine density increased. The spine morphology changed, and at the age of 7 months the majority of them achieved their morphological shape (with a shorter stalk compared to the early stages of development) (Figure 1). Hair-like spines had disappeared already at the age of 2.5 postnatal months.

No abnormal dendritic protrusions in both the control and Down syndrome specimens were noticed. It was not possible to notice qualitatively any other changes which could indicate a disruption in the dendritic and dendritic spine development in DS cases compared to the close age matched control subjects (Figures 1, 2). The qualitative analysis led us to the conclusion that DS subjects follow the normal developmental curve at least up to the 4.5th postnatal month.

3.2 Quantitative analysis

Quantitative parameters of dendritic and dendritic spine development did not show any major differences in development between the Down syndrome and control infants, neither between basal nor oblique dendritic tree.

The outgrowth of primary branches occurred up to age of 36 pcw for basal, and up to the age of first postnatal month for oblique dendrites (Figure 3A). The segment outgrowth, as the elongation of dendrites occurred in the period between 36 pcw and the first postnatal month for both analysed parts of the dendritic tree (Figure 3B,C). Before and after this period no significant segment outgrowth, nor dendritic elongation were observed.

Since the criteria for selection of neurons for quantitative analysis were based on the position of basal dendritic tree, significantly higher values of total number of segments as well as the length of the oblique dendritic tree at the age of 1 postnatal month were interpreted as a result of methodological factors (Figure 3C). We suppose that in the period between 1 and 2.5 postnatal months large apical dendritic elongation occurs, so that the origin of the oblique dendrites becomes more distant from the cell body, and therefore at later stages more oblique dendrites originate in the part of the apical dendrite outside the analyzed section. The data of individual segment length (mean terminal and intermediate) for oblique dendrites did not show any changes after the time when the final dendritic complexity was reached (first postnatal month), supporting our previous interpretation (Figure 3D,E).

Also, no significant changes were observed for individual segment length of basal dendrites (Figure 3D,E). There were 3-4 times more segments per dendrite in the basal versus the oblique dendritic tree (Figure 3B). The mean length of the individual terminal segments did not differ between the basal and oblique tree (Figure 3D), but the mean length of the intermediate segments was 3 times greater on the oblique dendrites (Figure 3E). Segment outgrowth and elongation did not differed in Down syndrome compared to control subjects.

Changes in the dendritic spine density indicated a continuous increase in the spine number after 36 pcw (Figure 4A,B). This increase was more pronounced up to the fourth postnatal month. The spine density showed close values on the terminal segments of both the basal and oblique dendritic tree (Figure 4B), as well as on the terminal segments of the oblique dendrites (Figure 4C). Values of spine density on the basal intermediate segments were 2-5 times lower compared to the spine density on the terminal segments of the same age (Figure 4C,D). This indicates that during the analyzed period only 5-10% of the dendritic spines on the basal dendritic tree were located on the intermediate

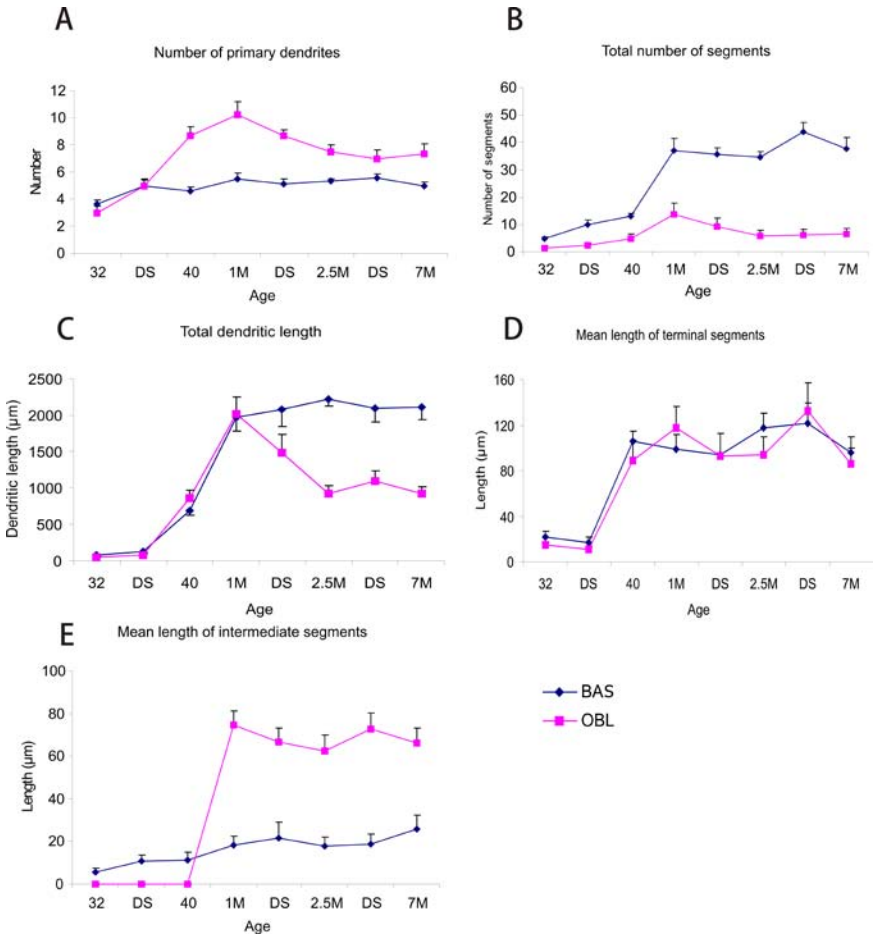

Figure 3. Three-dimensional analysis of basal and oblique apical dendrites. Number of primary dendrites (A); total number of segments (B); total dendritic length (C), mean length of terminal segments (D); and mean length of intermediate segments (E). All values expressed as mean ± SEM. Abbreviations: M. postnatal months; DS, Down syndrome; BAS, basal dendrites; OBL, apical oblique dendrites.

segments. The spine density on the intermediate segments of the basal dendrites of the 4.5 months old Down syndrome subject revealed at least 4 times lower values compared to the other subjects (Figure 4D). However, there was no statistical significance among them because of the extremely huge standard deviation in all the analyzed subjects. In addition, in general very low spine density was present on the intermediate segments of the basal dendritic tree (Figure 4D).

With respect to the intermediate segments on the basal dendrites, on all the presented parameters of spine density, it was visible that the Down syndrome subjects aged 2.5 and 4.5 pm had a much larger standard deviation compared to the control subjects aged 1 and 2.5 postnatal months (Figure 4C,D). Values of spine density on the total dendritic tree showed an absence of any significant increase between the control subject aged 2.5 postnatal month and 4.5 months old Down syndrome subject, on both the basal and oblique dendritic tree (Figure 4B). On the oblique dendrites there was also an absence of any increase in the spine density between the 1 month old control subject and the 2.5 months old Down syndrome subject, and the 2.5 months old Down syndrome subject showed significantly lower values compared to the 2.5 months old control subject (Figure 4B).

The discrepancies present in Down syndrome subjects were not observed when comparing the spine density separately on the intermediate and terminal segments (Figure 4C,D). Also, an increased standard deviation was present in the control subject aged 7 postnatal months. Therefore, it is difficult to conclude whether the present changes are the first signs of fine disturbances in the spine formation of Down syndrome subjects, or if they only represent inter-individual differences independent of pathological changes.

4. Discussion

The results of this study provide evidence that the development of dendrites and the formation of dendritic spines in fetuses and infants with DS proceed in a normal course, at least until the age of the 4.5 postnatal months (pm). Nevertheless, our data cannot completely exclude the possibility that the slight delay in spine formation is already present in DS infants at the age between 2.5 and 4.5 pm.

We also found that during the entire analyzed period (32 pcw – 7 pm), rapid and significant growth of the dendritic tree is present only during a limited period of 2 months (36 pcw–1 pm), which is in agreement with previously published findings on the normal development of the basal dendritic tree of the prefrontal layer IIIc pyramidal neurons [21,32,33,40,43,44]. These data also suggest that during prenatal development all primary dendrites are grown, so that the dominant event responsible for dendritic tree elongation, during this rapid stage of growth, is an outgrowth of new branches (segments). Actually, in previous studies of the basal dendritic tree development of the layer IIIc pyramidal neurons, it was shown that after the age of 1 postnatal month there was no significant increase in the number of segments up to adult age, and that at the age of 2.5pm the basal dendrites had achieved their final orientation [32], Additional elongation of the dendritic tree, without significant segment outgrowth, of the layer IIIc neurons occurs later during childhood, around the age of 2 years [32], Our data support these findings, and in addition revealed that the changes in the basal and oblique dendrites up to the postnatal age of 7 months occur synchronously, indicating that these morphologically different parts of the dendritic tree of neocortical neurons were functionally similar [39,48]. Changes in the dendritic spine density also occurred in parallel on both parts of the dendritic tree, showing a large increase during the postnatal time examined, with numerous filopodia-like processes during the period of intensive dendritic growth.

The most common problem in evaluating quantitative data from the Golgi studies is a limitation of the Golgi procedure on postmortal tissue. Therefore, we performed the rapid Golgi method, which is preferred to other Golgi methods, in order to completely impregnate the neuron fine structures (*i.e.* dendritic spines,

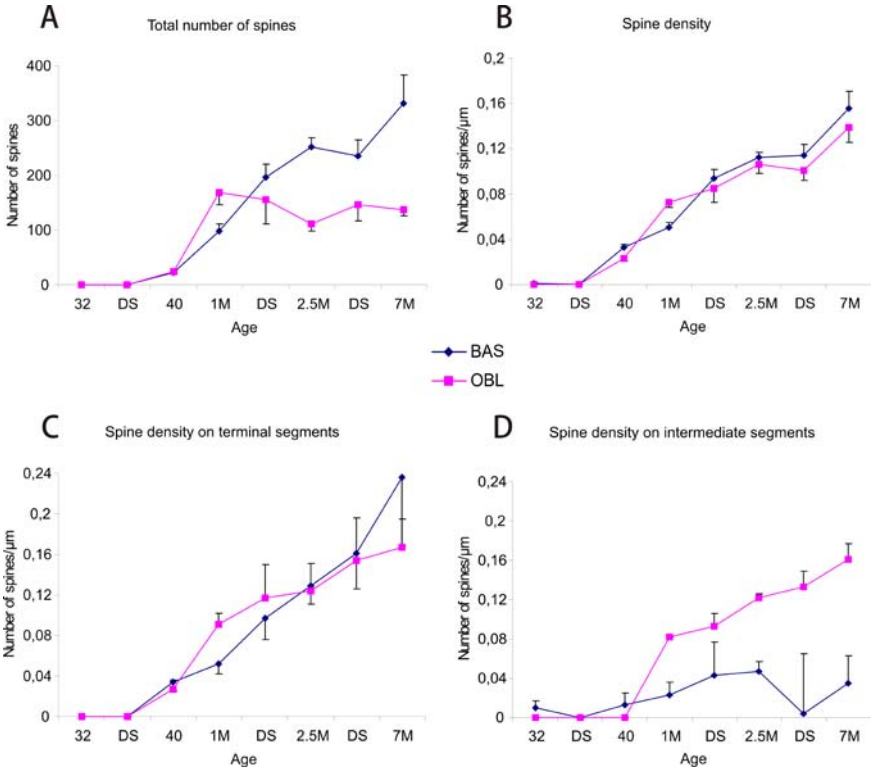

Figure 4. Three-dimensional analysis of spines on the basal and oblique apical dendrites. Total number of spines (A); spine density (B); spine density on terminal segments (C), spine density on intermediate segments (D). All values expressed as mean ± SEM. Abbreviations: M. postnatal months; DS, Down syndrome; BAS, basal dendrites; OBL, apical oblique dendrites.

the thinnest terminal part of the dendritic tree), and as the method with special ability to stain the large neurons in the deep part of the layer III, which were the target of our study [49]. Since the postmortem time was short and without any preagonal state, the quality of the sections was at the highest level. Therefore, it is reasonable to conclude that we minimized influence of methodological factors on the obtained results [50]. Since the normal developmental curve corresponded to data of previous independent studies which had partially analyzed the same period as in our study [32,43,44], we concluded that the present pattern of growth is typical for the normal development of the large layer IIIc pyramidal neurons. In the interpretation of our results we only had to respect the possibility that the well-preserved neurons in DS infants had a higher tendency to be impregnated, and because of that they tended to be selectively included in the quantitative analysis. Since the qualitative evaluation did not support this possibility, we strongly suggest that the vast majority of the layer IIIc pyramidal neurons in DS infants undergo the normal dendritic and dendritic spine development until the age of 4.5 postnatal months.

Layer IIIc pyramidal neurons are one of the key neuronal elements in processing higher cognitive functions [28,29,34,38,51]. It is interesting that there has been no full study done on the development of the pyramidal neurons in the associative cortices in children with DS. Previously, reports have focused only on other regions of the cerebral cortex (primary sensori-motor or primary visual). Our findings are consistent with other results that the pyramidal neurons in children with DS develop normally up to the fourth postnatal month. Later on, atrophy of the basal dendritic tree, as well as some morphologic alterations of their dendritic spines occur. Other studies have shown reductions in the number of dendritic spines on the apical dendrites in different regions of the cortex [14,23]. In line with such findings, it must be mentioned that there are numerous studies indicating well-preserved brains in children with DS during the perinatal period (or at the time of birth); for example: myelination [52]; brain gross morphology [8]; morphometric analyses of the skull [53]. It is especially interesting that cholinergic basal forebrain, which abundantly innervates layer IIIc pyramidal neurons, displays also no abnormalities during this early postnatal period [54,55].

However, we cannot simply conclude that associative layer III pyramidal neurons undergo dendritic atrophy immediately after the age of 4.5 postnatal months. During our analysis, these neurons normally achieved only around 60% of their later childhood and adult values [32], and the number of spines is more than half of that compared to maximal values achieved during childhood and still fewer than the spine number in the adult subjects [33]. This additional dendritic elongation, parallel with the increase in spine number, will occur mostly later, around the age of 2 years [32,33]. Therefore it is possible that there is a disturbance in dendritic and spine development of the associative layer IIIc pyramidal neurons in DS subjects later during childhood, and that this could lead to lower values of the dendritic length and spine number in adulthood. In contrast, it is also possible that the dendritic and spine development of the associative layer IIIc pyramidal neurons in DS children are not seriously affected, nor later on during childhood. Although the later development of the associative layer IIIc pyramidal neurons is a subject of pure speculation, only fully and timely development of these neurons represents a prerequisite for the normal cognitive functions later in life [37]. It must also be added that the associative layer IIIc pyramidal neurons are selectively vulnerable in some diseases characterized by cognitive decline and the rate of their lost parallels with the rate of the cognitive decline in these patients [56-59]. Considerable variability of intellectual function has been reported among individuals with DS [60]. A positive influence of environmental stimulation on cognitive development of children with DS has also been previously noticed [61]. In addition, children with DS who grew up at home had much better preserved intellectual abilities than those who were raised in orphanages [62,63]. Although it is based on an indirect conclusion, it is very intriguing to speculate if the development of the associative layer IIIc pyramidal neurons was highly preserved through to the later development in DS patients, would it also preserve the cognitive and intellectual abilities? Since we obtained parameters of the normal development during the whole postnatal stage, we intend to answer this question in the future, but this research is highly unpredictable according to the access of the post-mortem DS material.

In this paper we showed that the layer IIIc pyramidal neurons in DS had achieved their final dendritic complexity (branching pattern) and established adult like dendritic orientation [32]. We also found that this stage of the dendritic development was characterized by proper spine production, so that all prerequisites for the normal development of the key neuronal elements in cortico-cortical circuitries are present in DS infants at the time of 4.5 postnatal months. These early developmental events seem not to be highly dependent on environmental influence, in contrast to later dendritic and dendritic spine development [39]. Therefore, it seems reasonable to conclude that the application of some therapeutical procedures in the infancy and childhood could mitigate or stop cognitive decline present later in patients with DS.

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