RESEARCH ARTICLE

Synaptic development of layer V pyramidal neurons in the prenatal human prefrontal neocortex: a Neurolucida-aided Golgi study

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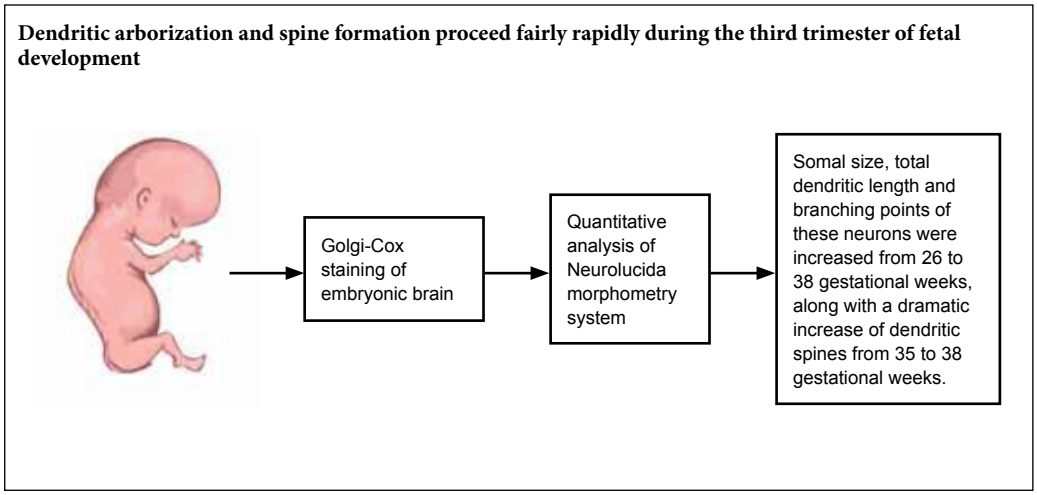
Graphical Abstract



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Abstract

The prefrontal neocortex is involved in many high cognitive functions in humans. Deficits in neuronal and neurocircuitry development in this part of the cerebrum have been associated with various neuropsychiatric disorders in adolescents and adults. There are currently little available data regarding prenatal dendrite and spine formation on projecting neurons in the human prefrontal neocortex. Previous studies have demonstrated that Golgi silver staining can identify neurons in the frontal lobe and visual cortex in human embryos. In the present study, five fetal brains, at 19, 20, 26, 35, and 38 gestational weeks, were obtained via the body donation program at Xiangya School of Medicine, Central South University, China. Golgi-stained pyramidal neurons in layer V of Brodmann area 46 in fetuses were quantitatively analyzed using the Neurolucida morphometry system. Results revealed that somal size, total dendritic length, and branching points of these neurons increased from 26 to 38 gestational weeks. There was also a large increase in dendritic spines from 35 to 38 gestational weeks. These findings indicate that, in the human prefrontal neocortex, dendritic growth in layer V pyramidal neurons occurs rapidly during the third trimester of gestation. The use of human fetal brain tissue was approved by the Animal Ethics Committee of Xiangya School of Medicine, Central South University, China (approval No. 2011-045) on April 5, 2011.

Key Words: *Golgi staining; human brain banking; neurodevelopment; Neurolucida; neuropsychiatric disorders; prefrontal cortex; synaptogenesis; three-dimensional reconstruction*

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Introduction

The prefrontal cortex is located in the anterior frontal part of the cerebrum and, evolutionarily speaking, it is one of the newest and most expanded brain structures (Teffer and Semendeferi, 2012; Donahue et al., 2018; Medina et al., 2019; Putt et al., 2019). This forebrain region can be divided into the ventromedial, dorsolateral, and orbitofrontal subdivisions, and their laminar and cellular organizations are characteristic of the associative neocortex. Each of these regions contains a number of Brodmann areas; for example, area 46 is located around the central area on the lateral surface of the frontal lobe (Brodmann, 1994; Mai et al., 2008; Ding et al., 2016). The prefrontal cortex has fundamental roles in many of the so-called high cognitive functions, including attention focusing, motivation and problem solving, decision making, and social interactions (Goldman-Rakic, 1996; Ridderinkhof et al., 2004; Boros et al., 2017; Daly et al., 2019; Fung et al., 2019; Hao et al., 2019; Sakurai and Gamo, 2019; Soltani and Izquierdo, 2019). Some prefrontal cortical areas, including Brodmann area 46, are considered critical for the formation of short-term memory (Petrides and Pandya, 1999) or working memory (Andrews et al., 2011), which are essential for cognitive processes such as reasoning, decision-making, and behavioral manifestations (Sakagami and Watanabe, 2007).

Neuropathological changes in the prefrontal cortex in development and in adulthood have been related to a number of neurocognitive disorders (Barbalat et al., 2009; Shalom, 2009; Teffer and Semendeferi, 2012; Dong and Feng, 2019; Fan et al., 2019). For example, attention-deficit/hyperactivity disorder is associated with reduced frontal lobe volume in children and adolescents (Mueller et al., 2017; Noordermeer et al., 2017; Jacobson et al., 2018; Ronel, 2018; Albaugh et al., 2019). In autism spectrum disorders, there is early aberrant brain overgrowth in the prefrontal cortex, as evidenced by abnormal laminar cytoarchitecture and cortical neuron disorganization in children with autism (Wegiel et al., 2010; Courchesne et al., 2011; Stoner et al., 2014; Donovan and Basson, 2017). In addition, the morphological maturation of pyramidal neurons in layer III of the prefrontal cortex is protracted during prenatal development and childhood in these disorders (Hutsler and Casanova, 2016; Petanjek et al., 2019). The excess neurons and delayed synaptic trimming may cause perturbed intra- and inter-cortical wiring, which may underlie the abnormal social, emotional, and communication functions that are manifested clinically in autistic children (Kumar et al., 2019). Moreover, deficient neuronal development in the prefrontal cortex may relate to the pathogenesis of schizophrenia (Goldman-Rakic and Selemon, 1997; Volk and Lewis, 2014). Specifically, abnormal development in the glutamatergic, GABAergic, and dopaminergic systems in the dorsolateral prefrontal cortex may cause an imbalance between excitatory and inhibitory neuron activities in schizophrenia (Laviolette, 2007; Hoftman et al., 2017; Ferguson and Gao, 2018).

To date, there are limited published data regarding neurogenesis and neuronal differentiation and maturation in the human neocortex. Several studies have shown the development of principal neurons and interneurons in the prenatal human frontal and visual cortices using the Golgi silver staining technique and immunohistochemical methods (Mrzljak et al., 1988; Yan et al., 1992, 1996; Cao et al., 1996). Neurons in the embryonic marginal zone (future layer I) and those in the subplate (future subcortical white matter) appear early, at 13.5–15 gestational weeks (GW) (Mrzljak et al., 1988; Yan et al., 1992, 1996). This development is followed by the differentiation of principal neurons and interneurons in the cortical plate (prospective layers VI–II) from 17–25 GW. In prospective layers III and V in the prefrontal cortex, dendritic development and spine formation in pyramidal neurons occur largely after 26 GW (Mrzljak et al., 1988, 1992). Here, we report morphometric data for somal growth, dendritic development, and spine formation in layer V pyramidal cells in the human prefrontal neocortex from mid- to full-term prenatal stages, based on Golgi–Cox staining and Neurolucida-based quantification.

Materials and Methods

Fetal brain specimens

Brain specimens were obtained via the body donation program at Xiangya School of Medicine, Central South University, China, following informed consent from the parents (Yan et al., 2015; Xu et al., 2019). At autopsy, five fetal brains were available with short postmortem delays of less than 2 hours, making them suitable for the assessment of normal neuronal morphology (**Table 1**). Causes of death included spontaneous abortion and induced abortion as a result of maternal complications, and there was also a neonatal case that involved a sudden infant death. Fetal ages, in GW, were determined in reference to maternal menstrual history as well as fetal body weight and fetal crown–rump length. The use of brain tissue was approved by the Ethics Committee of Xiangya School of Medicine (approval No. 2011-045) on April 5, 2011. The temporal lobes of the brains were used in a previous Golgi silver stain study that characterized the development of soma, dendrites, and dendritic spines in CA3 pyramidal neurons (Lu et al., 2013).

Rapid Golgi-Cox staining

After removing the brain from the skull, a 2 cm thick cerebral slice was blocked from the middle part of the frontal lobe. Rapid Golgi–Cox staining of the tissue was conducted using a commercial staining kit (FD NeuroTechnologies,

Table 1 Postmortem brain characteristics

Case	Age (gestational week)	Body weight (g)	Sex	Cause of death
1	19	308	Male	Spontaneous abortion
2	20	328	Female	Teenage pregnancy, induced abortion
3	26	1025	Female	Teenage pregnancy, induced abortion
4	35	2650	Male	Maternal accident
5	38	2820	Female	Teenage pregnancy, accident fetal death

Ellicott City, MD, USA). After Golgi impregnation, brain blocks were sectioned at 100 μm thickness, mounted on gelatin-coated glass slides, and allowed to air-dry in the dark. Sections were then dehydrated, cleared, and coverslipped using a commercial OTC mounting medium (Electron Microscopy Sciences, Hatfield, PA, USA).

Golgi-impregnated sections were examined on a microscope equipped with the Neurolucida three-dimensional system (MBF Bioscience China, Hong Kong, China). In each section, ten pyramidal neurons from layer V were constructed at 40× magnification for quantitative analysis, in accordance with the software developer's instructions. Neurons were selected based on the following criteria: (1) they were located around the middle region of layer V; (2) they were among the labeled neurons with the widest dendritic fields; (3) the somatic and dendritic processes were distinctly visualized; and (4) they were relatively distant from other silver-stained cells. The following measurements were obtained: somal area, as well as the total lengths of apical and basal dendrites, branching nodes (or points) and spine density (per 10 μm dendritic length) of the apical and basal dendrites. For each brain, 20 pyramidal neurons were quantified.

Statistical analysis

Data are expressed as the mean ± SEM, and were statistically analyzed using one-way analysis of variance followed by Bonferroni *post hoc* tests (GraphPad Prism 4.02, GraphPad Software, San Diego, CA, USA). A value of $P < 0.05$ was considered statistically significant.

Results

Overall morphological development of layer V pyramidal cells

Consistent with previous studies (Yan et al., 1992, 1996, 1997), in the fetal brains at 19 GW and 20 GW, the deep cortical layers (VI and V) were already differentiated in the cortical mantle of the frontal cerebrum, while the remaining layers (IV–II) of the gray matter remained part of the developing cortical plate and were therefore not distinguishable. In Golgi-impregnated sections from the brains at 19 GW (**Figure 1A–D**) and 20 GW (**Figure 1E–H**), silver-stained neuronal profiles were observed in the deep region of the cerebral mantle, corresponding to layers V and VI. These neuronal profiles were predominantly bipolar or fusiform, with a long somal axis oriented perpendicular to the pia. Two major dendrite-like processes were often observed on each soma, with an apical process running towards the pia matter and a basal process oriented towards the lateral ventricle (**Figure 1B–D** and **F–H**). At high magnifications, small protrusions were present on the soma and along dendritic processes, especially on their proximal parts (**Figure 1C** and **D**). In the 20 GW brain, some Golgi-impregnated neurons had more than two processes, indicative of ongoing dendritic branching (**Figure 1F** and **G**). Spines with a long stem or a thin neck were increasingly observed on the proximal dendritic regions among some Golgi-stained neurons in this brain, suggesting the ongoing formation of morphologically complex spines (**Figure 1G** and **H**).

In the 26 GW fetal brain (**Figure 1I–J**), Golgi-stained neurons in layer V had longer dendritic processes compared with those in the 19 GW and 20 GW cases. The soma of these neurons were mostly pyramidal, with thin apical dendrites running towards the pial surface. Most of the pyramidal neurons had more than one basal dendrite, while dendritic branches were clearly observed on both the apical and basal dendrites. Spines were relatively densely packed along the proximal parts of the apical and basal dendrites (**Figure 1K** and **L**).

In the 35 GW and 38 GW fetal brains, Golgi-impregnated neurons in layer V were typically pyramidal (**Figures 1M–P** and **2A** and **2B**). Apical and basal dendrites could be distinctly visualized and extended over long distances (**Figures 1N** and **2A** and **2B**). Multiple basal dendrites were observed on most layer V pyramidal neurons, extending from the lower half as well as the basal aspect of the soma. Branches on apical and basal dendrites were also clearly labeled (**Figures 1N–P** and **2A–F**). An axon-like process could be identified on most Golgi-impregnated pyramidal neurons, arising at approximately the middle point of the somal base (**Figures 1N** and **2B**). In these two brains, somal and dendritic spines on layer V pyramidal cells also exhibited complex morphologies. Thus, at high magnification, most spines appeared to be dot-like features aligned along the dendritic shaft or somal surface. Some spines were connected to the dendrites with a short neck, while others appeared to be long-necked spines (**Figures 1O**, **1P** and **2B**).

Quantitative developmental data from layer V pyramidal neurons

Growth in somal size in layer V pyramidal neurons

Results of one-way analysis of variance revealed that there was an overall difference in somal size among the different cases ($P < 0.0001$; $F = 1226$; $df = 4$). Specifically, the Bonferroni multiple comparison test indicated that there were differences in somal size between the 19 GW and 20 GW cases and cases of other gestational ages, as well as between the 35 GW and 38 GW cases (**Figures 2D** and **3A**).

Dendritic growth and arborization in layer V pyramidal neurons

One-way analysis of variance results indicated an overall difference in dendritic length among the different cases ($P < 0.0001$; $F = 1311$; $df = 4$). *Post hoc* testing indicated that there were significant differences between the gestational ages, except when comparing the 36 GW *versus* 38 GW cases (**Figure 3B**).

There was a statistically significant overall difference in the average total length of basal dendrites among the different cases ($P < 0.0001$; $F = 1352$; $df = 4$). *Post hoc* testing indicated that there were significant differences between the different gestational ages (**Figure 3B**).

There was also a statistically significant overall difference in the average number of nodes on apical dendrites among the cases ($P < 0.0001$; $F = 5655$; $df = 4$). *Post hoc* analysis revealed significant differences between the different gestational ages.

Furthermore, one-way analysis of variance testing indicated that there was an overall difference in the numbers of nodes on basal dendrites among the cases ($P < 0.0001$; $F = 2019$; $df = 4$). Moreover, *post hoc* analysis revealed statistically significant differences between the different gestational ages, except when comparing the 19 GW *versus* 20 GW cases (**Figure 3C**).

Formation of dendritic spines on layer V pyramidal neurons

The average spine densities on apical dendrites were 0 ± 0, 0 ± 0, 2.3 ± 0.36, 7.8 ± 2.97, and 9.1 ± 3.08 in the fetal brains at 19, 20, 26, 35, and 38 GW, respectively (**Figure 3D**). One-way analysis of variance testing revealed an overall difference in means among the different gestational ages ($P < 0.0001$; $F = 102$; $df = 4$). *Post hoc* analysis revealed no differences between the 19 GW and 20 GW brains or between the 35 GW and 38 GW brains, but there were significant differences between the other gestational ages. One-way analysis of variance testing indicated an overall difference in the average spine densities of basal dendrites among the different gestational ages ($P < 0.0001$; $F = 96.8$; $df = 4$). Specifically, there were differences between the different gestational ages, except when comparing the 19 GW versus 20 GW cases (**Figure 3D**).

Discussion

As mentioned in the introduction, several previous studies have addressed neuronal development in the human prefrontal neocortex (Mrzljak et al., 1988, 1992; Koenderink et al., 1994; Yan et al., 1996; Sedmak et al., 2018). An early Golgi study examined neuronal development in the dorsolateral and lateral prefrontal cortices in humans from 13.5 GW up to 2 months of age (Mrzljak et al., 1992). The basal dendrites of layer III and V pyramidal neurons grow slowly during the first two-thirds of gestation, followed by a rapid increase between 27–32 GW. The average number of basal dendrites per pyramidal neuron appears to stabilize at around 26–27 GW. The somal and dendritic development of subplate neurons occurs earlier than that of cortical pyramidal neurons (Mrzljak et al., 1992). Another previous Golgi-Cox study reported dendritic development in layer V pyramidal neurons in the human prefrontal cortex from infancy to adulthood (the oldest subject was in their 30s) (Koenderink and Uylings, 1995). This study reported that the growth of dendrites proceeds rapidly during the first postnatal year, and this continues at a reduced rate up to around 5 years of age. After this age, dendritic lengths tend to stabilize until approximately 27 years of age. A more recent study reported a biphasic pattern of dendritic development in layer IIIC magnopyramidal neurons in humans (Sedmak et al., 2018). The basal and oblique dendrites of these large pyramidal neurons grow rapidly during the early postnatal months, which is associated with dendritic branching. After a "dormant" period (from 2.5 to 16 months of age), followed by a second phase of outgrowth until approximately 3 years of age, these dendritic structural changes are considered to be related to the development of cognitive functions during early childhood.

In the present study, somal size in prefrontal layer V pyramidal neurons increased more than threefold (from 105.1 ± 10.3 μm^2 to 357.9 ± 21.7 μm^2) from the middle to late terms of gestation, while the growth in somal size occurred most rapidly from 26 to 38 GW. Along with the increase in somal size, apical and basal dendritic length was also greatly increased from 26 to 38 GW, with the former approaching nearly 400 μm in the oldest fetal brain. In addition, arborization occurred in the apical and basal dendrites, as indicated by an increased number of branching points; there were approximately 7 and 5 nodes on the apical and basal dendritic trees in the fetal brain at 38 GW. Overall, these findings indicate that, in principal neurons of the human prefrontal neocortex, somal and dendritic development proceeds rapidly in the third trimester of gestation.

The Neurolucida-based quantification of dendritic spines in the present study revealed that few spines were large enough to be recognized by the computer program in the fetal brains at 19 and 20 GW; however, visual microscopic inspection indicated that some small dendritic protrusions resembling spines had already appeared on developing dendrites in these cases. Morphologically well-defined spines were clearly seen on layer V pyramidal neurons by 26 GW, and the densities of dendritic spines increased significantly from 26 GW to 35 GW and 38 GW. These results suggest that spine formation progresses rapidly during the last trimester of gestation, along with dendritic development. It should be noted that, because of the limited number of cases that were available for the present study, the data presented in the current study only reflects an overall trend of early morphogenesis in layer V pyramidal neurons in the human prefrontal neocortex. However, when the present results are taken together with the data reported in previous postnatal studies (Koenderink and Uylings, 1995; Sedmak et al., 2018), it appears reasonable to conclude that dendritic development (including spine formation) on projecting neurons in the human prefrontal neocortex occurs rapidly during the last prenatal month and continues throughout infancy and childhood, orchestrating the development of high cognitive functions.

Taken together, the present Golgi–Neurolucida investigation of the prenatal prefrontal cortex revealed that somal development and dendritic and spine formation in layer V pyramidal neurons occur largely after the mid-term of gestation. Dendritic arborization and spine formation proceed relatively rapidly during the third trimester of fetal development.

Author contributions: *Study design, experiment implementation and manuscript writing: LXH, DHL; study design and manuscript writing: LXH, DHL; data analysis: WX, JML, LW, AHP. All authors approved the final version of the paper.*

Conflicts of interest: *The authors declare no competing interests.*

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Institutional review board statement: *Postmortem brains were used following proper consent from the patients and this study met all require-*

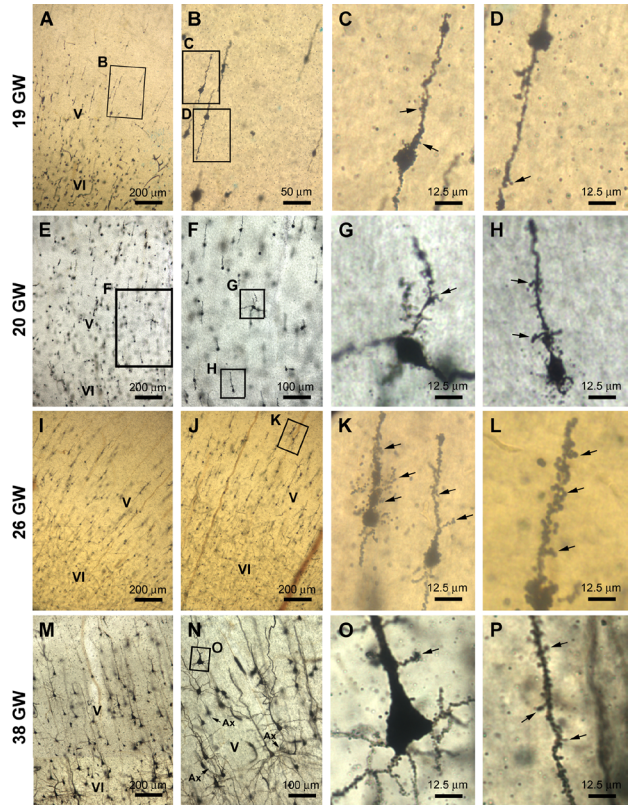

Figure 1 Golgi-stained neuronal profiles in the prenatal human frontal neocortex.
The cases, prenatal ages expressed as gestational weeks (GW), and cortical lamination (layers V, VI) are as indicated. The two left panels show low-magnification images, with enlarged views shown on the right as indicated. Golgi-impregnated layer V neurons in the 19 and 20 GW fetal brains were largely bipolar, with an apical and a basal dendrite oriented vertically in the developing cortex (A–H). Dendritic spines (arrows) were observed on the dendrites; some had a long, thin long neck (G, H). In the 26 GW case (I, J), some silver-stained layer V neurons appeared pyramidal (K), with spines densely packed on the proximal (K) and distal (L) segments of apical dendrites. In the 38 GW fetal brain, the Golgi-stained neurons in layer V were large pyramidal cells (M, N) with distinct apical and basal dendrites. Axons (Ax) were also clearly observed at the base of the neuronal soma (N). Spines with complex morphologies were present on proximal (O) and distal (P) dendritic processes. Scale bars in the individual image panels are as indicated.

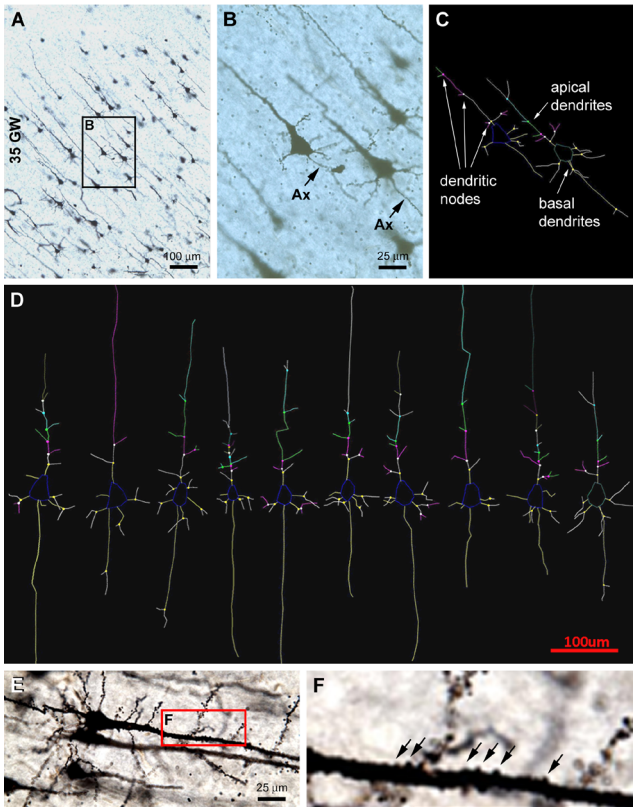

Figure 2 Neurolucida-aided quantitative analysis of Golgi-stained neurons in the prenatal human frontal neocortex.
(A, B) Low-magnification images, high-magnification images, and representative cell drawings of layer V neurons in the 35 GW fetal brain. Pyramidal neurons had distinctly labeled apical and basal dendrites as well as axons (Ax) (B). (C) Cell drawings of two layer V pyramidal neurons from (B), denoting the dendritic segments (marked in different colors) and branching nodes (colored dots) of the apical as well as basal dendrites. (D) Variability of 10 Neurolucida-marked pyramidal neurons from the 35 GW case. (E, F) Dendritic spines (arrows) on a pyramidal neuron.

ments and regulations set by the Animal Ethics Committee of Xiangya School of Medicine, Central South University, China (approval No. 2011-045) on April 5, 2011.

Informed consent statement: *The authors certify that they have obtained all appropriate consent forms from the embryo donors. In the forms, the parents have given their consent for the images and other clinical information to be reported in the journal. The parents understand that their names and initials will not be published.*

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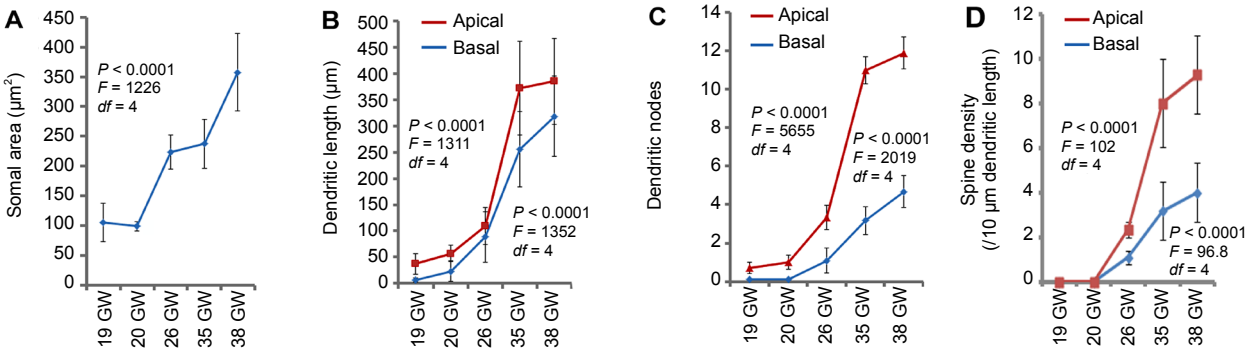

Figure 3 Trends of changes in somal and dendritic parameters in Golgi-stained layer V pyramidal neurons in the prenatal human frontal neocortex based on Neurolucida-based morphometry.

(A) Means and ranges of the somal sizes of neurons in individual cases. There was an overall difference in somal size between the different cases ($P < 0.001$, $F = 1226$, $df = 4$). (B) Total mean lengths of the apical and basal dendrites, and the ranges of these dendrites, obtained from the prenatal brains. There was an overall difference in the total mean length of apical ($P < 0.001$, $F = 1331$, $df = 4$) and basal ($P < 0.001$, $F = 1352$, $df = 4$) dendrites among different cases. (C) Means and distribution ranges of the apical and basal dendritic nodes. There was an overall difference in the dendritic nodes of the apical ($P < 0.001$, $F = 5655$, $df = 4$) and basal dendrites ($P < 0.001$, $F = 2019$, $df = 4$) among different cases. (D) Means and ranges of spine densities of the apical and basal dendrites in the different cases. There was an overall difference in the spine densities of the apical ($P < 0.001$, $F = 102$, $df = 4$) and basal ($P < 0.001$, $F = 96.8$, $df = 4$) dendrites among the different cases. Data are expressed as the mean ± SEM. One-way analysis of variance indicated an overall difference for all four measurements with the increase in gestational weeks (GW).

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