Transient Cellular Structures in Developing Corpus Callosum of the Human Brain

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ABSTRACT

The corpus callosum connects two cerebral hemispheres as the most voluminous fiber system in the human brain. The developing callosal fibers originate from immature pyramidal neurons, grow through complex pathways and cross the midline using different substrates in transient fetal structures. We analyzed cellular structures in the human corpus callosum on postmortem brains from the age of 18 weeks post conception to adult, using glial fibrillary acidic protein, neuron-specific nuclear protein, and chondroitin sulphate immunocytochemistry. We found the presence of transient cellular structures, callosal septa, which divide major fiber bundles and ventrally merge with subcallosal zone forming grooves for callosal axons. The callosal septa are composed of glial fibrillary acidic protein reactive meshwork, neurones and the chondroitin sulphate immunoreactive extracellular matrix. The developmental window of prominence of the callosal septa is between 18-34 weeks post conception which corresponds to the period of most intensive growth of callosal axons in human. During the early postnatal period the callosal septa become thinner and shorter, lose their neuronal and chondroitin sulphate content. In conclusion, transient expression of neuronal, glial and extracellular, growing substrate in the callosal septa, as septa itself, indicates their role in guidance during intensive growth of callosal fibers in the human brain. These findings shed some light on the complex morphogenetic events during the growth of the corpus callosum and represent normative parameters necessary for studies of structural plasticity after perinatal lesions.

Key words: human, development, commissural pathways, subcallosal zone

Introduction

The corpus callosum (CC) is the most voluminous cerebral fiber system in the human brain. In man, the CC reaches the maximum size and complexity of all mammals. In the primate brain, fibers of the corpus callosum originate predominantly from layer III pyramidal neurons of the neocortex[1,2] while anatomical studies of experimental rodents have demonstrated that the majority of contralaterally projecting (callosal) neurons are located in cortical layers II/III and layer V[3].

The development of the corpus callosum is a complex event because callosal axons firstly surround the ipsilateral ventricular system and then turn medially across the midline and grow into the opposite hemisphere towards the homotypical target area, interacting with different cellular structures and the extracellular matrix (ECM)[4,5]. The development of the corpus callosum is a dynamic process that continues throughout gestation until well after birth.

In the experimental rodents, the early callosal growth is supported by several transient cellular structures situated along the midline. In rodents, the midline zipper glia (MZG), glial wedge (GW) and indusium griseum glia (IGG) have been shown to play critical roles in corpus callosum development[6,7]. These different midline structures have common phenotypic and molecular characteristics (e.g. expression of guidance molecule Slit2, which prevents callosal fibers from entering the septum, and instead guides them across the midline into the contralateral hemisphere) indicating that they may represent the same population of glia that becomes spatially distinct by the formation of the corpus callosum[7]. The other structure involved in callosal development is the midline (neuronal-glial) sling (MSL) situated at the corticoseptal border[6,8]. The significance of subcallosal and pericallosal structures for CC development has been highlighted by mice mutants with affected midline structures in which

the corpus callosum fails to form. Instead, the callosal axons grow into large ectopic fiber bundles on either side paramediosagittaly resembling the Probst's bundle (for review see Richards et. al. 2004)[5].

The morphogenesis and early growth of the CC in humans were described in the classical embryological studies (for review see Rakic and Yakovlev 1968)[4]. In human brain, the first callosal axons cross the mediosagittal plane around the eleventh week post conception (WPC) through transient structure of the medial telencephalon, designated as *massa commissuralis[4]*. Another morphogenetic midline structure is the subcallosal zone described in the human brain as a part of the subcallosal septohippocampal continuum (nucleus septohippocampalis)[9]. The majority of neuronal and glial elements in the subcallosal zone are transient, suggesting that they may be involved in the guidance of the callosal axons and bi-directional growth of fornix fibers. Recently, two groups[10,11] have shown that structural and molecular substrates regulating the early callosal development in humans are similar to those described in mice, indicating evolutionarily conserved mechanisms.

There are very few histological studies describing the later stages (after 24 WPC) of CC development. In the studies performed by Clarke et. al. a decrease of the cross-section callosal area size was observed between 32 WPC and the second postnatal month, suggesting the beginning of retraction of the callosal axons[12]. However, the experimental studies in developing monkey brains, which used quantitative analysis of axon numbers, have shown that the late fetal phases of CC development are characterized by significant, 3.5-fold overproduction of axons[13], suggesting reduction of exuberant axons and significant reorganization of projections at a later point[3]. The CC of adult monkeys contains finally around 56 million axons[13]. In monkey brain, the topographical organization seems to be established already by the 133rd day post conception[2], which is much earlier than the achievement of the final number of axons.

The interest for development of the CC has been increased recently due to the fact that the CC was found to be the most frequently damaged structure in perinatal hypoxic-ishemic brain lesions[14,15]. This finding is based on correlated MRI and measurements of cross-sectional analysis. It has also been shown that CC abnormalities accompany more then 90 clinical syndromes while agenesis of the corpus callosum (ACC) is associated with over 50 different human congenital syndromes[5,16].

Considering the clinical and pathological significance of the corpus callosum, its importance for cognitive functions[17,18] and its frequent lesions in the perinatal period[14] we have initiated a long term study of the callosal commissural pathways in men. The specific aim of this study is to analyze transient structures associated with the CC and to obtain cellular parameters specific for morphogenesis of the midline structures in the human brain. The normative data obtained in the present study will be used for correlation with neuroimaging studies and studies of structural rearrangements of commissural system following perinatal lesions.

Materials and Methods

Post mortal human brain tissue without macroscopically or microscopically discernible signs of neuropathological alteration was obtained from: (i) the fetuses spontaneously or medically (indicated) aborted, (ii) preterm infants, (iii) children and (iv) adult subjects, with a non-neurological cause of death (Table 1). The autopsies were approved by the Internal Review Board of the Ethical Committee at the School of Medicine, University of Zagreb in accordance with the Helsinki declaration from 2000. The fetuses, age ranged from 18th week post conception (WPC) to the newborn infants (estimated on the basis of their crown-rump length and/or the pregnancy records), were grouped in developmental phases on the basis of the major characteristics of developmental cortical organization (Table 1). Additional brain sections belonging to the Zagreb Neuroembryological Collection[19] were analyzed.

The brains were fixed (24h in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4), blocks of tissue embedded in paraffin and frontal, sagittal or horizontal sections were cut to 15 μm thickness.

To delineate the cytoarchitectonic boundaries and cellular compartments of the human fetal midline telencephalon, sections were processed with Nissl modification of cresyl-violet staining. To examine the location and relative regional abundance of the ECM molecules rich in sulphated glycosaminoglycans, and to examine content of neuronal and glial cells and their morphology in the midline structures, immunohistochemical labeling was performed, using the primary antibodies described in Table 2. The immunohistochemical procedure was performed as previously described[20].

The sections were analyzed with an upright microscope Olympus AX70, images captured with a digital camera Nikon DXM1200 or scanned with a Nikon Super Coolscan 9000 ED.

Results

In all examined stages, the corpus callosum was found as a well developed fibrillar structure composed of axonal bundles, which run in transverse, coronal planes towards the midline and after crossing the midline, curves along the roof and lateral wall of the lateral cerebral ventricles.

The outlines of the major callosal parts: genu, body and splenium, as described in classical literature[4], are clearly visible even in the youngest specimens examined.

On the midsagittal sections stained with Nissl or immunolabeled with glial fibrillary acidic protein (GFAP) numerous striations which incompletely divide callosum into irregular segments (Figure 1: A, B, C, D) are visible. For these, not yet described, structures we propose the term callosal septa because separates axonal bundles of

TABLE 1
RECORD OF ANALYZED BRAIN TISSUE

Developmental phases	Code	Gender	Age	Postmortem delay
Fetal phase (until 24 wpc)	CF542	female	18 wpc	12h
	CF543	male	18–20 wpc	7h
	CF475	male	19 wpc	12h
	CF541	male	19 wpc	12h
	CF480	female	20 wpc	4h
	CF479	male	20 wpc	4h
	CF526	male	20 wpc	16h
	CF520	female	21 wpc	8h
	CF471	male	21/22 wpc	9h
	CF545	male	22 wpc + 4 days	12h
	CF535	male	22 wpc + 2 days	12h
Early preterm phase (24–32 wpc)	CF537	female	24 wpc	4h
	CF546	male	25 wpc	8h
	CF517	female	26 wpc + 24 days	12h
Late preterm phase	CF492	male	35 wpc	24h
Newborn	CF477	male	40 wpc	7h
	CF528	male	40 wpc	17h
Infant	CD289	male	3 months	8h
Child	CD255	male	6 years	6h
Adult	CO340	male	42 years	6h

wpc – weeks post conception

TABLE 2
PRIMARY ANTIBODIES USED IN THE STUDY

Primary antibodies	Abbrev.	Host / isotype	Dilution	Supplier
Specific neuronal marker				
Anti Neuron-specific Nuclear protein	NeuN	monoclonal mouse IgG1	1:1000	Chemicon, Temecula, CA
Specific glial marker				
Anti Cow Glial Fibrillary Acidic Protein	GFAP	monoclonal rabbit IgG	1:200	DAKO, Copen-hagen, Denmark
Extracellular matrix marker				
Anti Chondroitin-Sulphate -56	CS-56	monoclonal mouse IgM	1:200	SIGMA, St. Louis, MO

the corpus callosum. The number of callosal septa was found to be individually variable, usually 15–20 thicker and longer septa and numerous smaller septa (Figure 1). The callosal septa stretch from subcallosal zone[9] towards dorsal aspects of corpus callosum. On the horizontal sections through the callosal corpus (cut transverse to the stiations), septa and striations look as a thin railway slippers on antero-posterior axis of the CC. On the coronal sections throughout CC, in ideally parallel cut to the septa they are not discernible but in the case when the cut is semioblique to the septa, structures look like very tiny flames arising from subventricular zone.

In addition, GFAP immunocytochemistry reveals the presence of thin radial striations of rather uniform sizes (Figures 1D and 2A, arrowheads), which continue into radial striations of the intermediate zone (Figure 1D).

The callosal septa enlarges in its ventral portion and merges with the subcallosal zone which laterally continues into the subventricular zone[9]. These two structures, callosal septa and subcallosal zone jointly form »grooves« in which callosal bundles are laid. The cellular content of septa can already be seen on Nissl preparations (Figure 1C). Different cellular populations can be distinguished with GFAP and neuron-specific nuclear protein (NeuN) immunolabeling. Callosal septa were pronounced throughout the second and third trimester of gestation (fetal and preterm phases) and they became thinner during postnatal months (Figure 2B).

We will describe the cellular composition and structural organization of these transient cellular structures during their developmental peak in fetus and preterm infants.
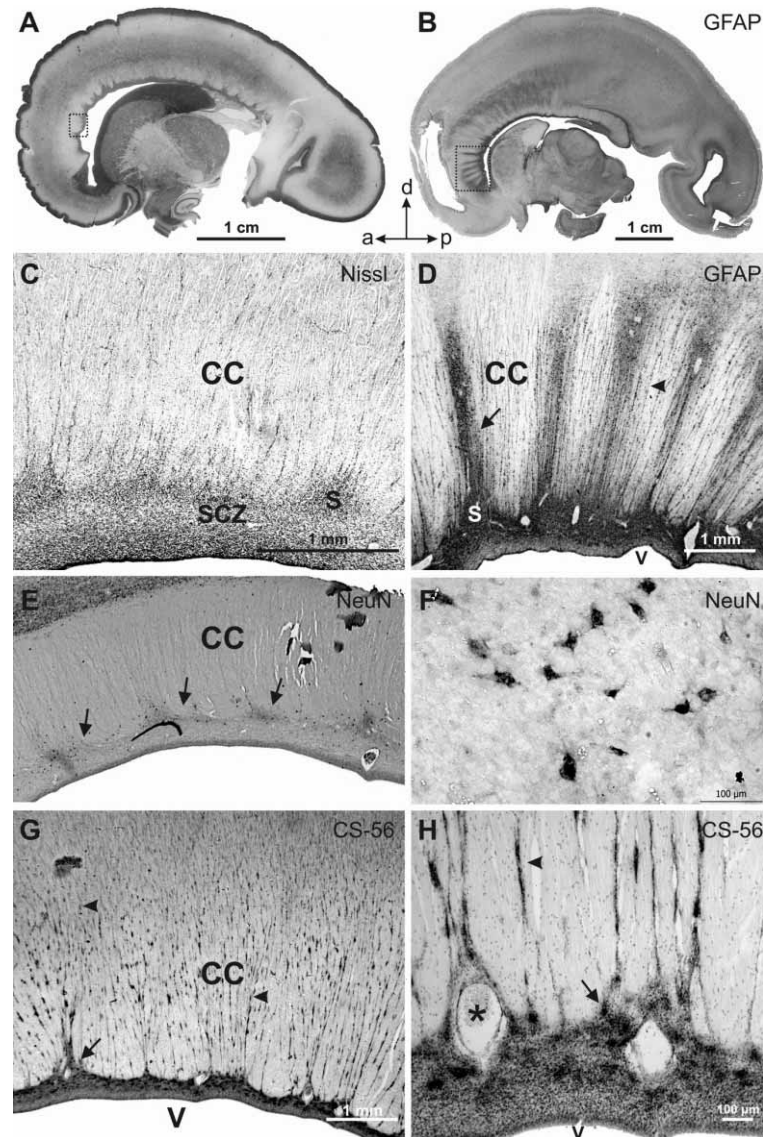
Fig. 1. Mediosagittal sections of the human fetal brain showing the transient cellular callosal septa at the 21 (A), 24 (E, F, G, H) and 25 (B, C, D) week post conception (WPC) stained with Nissl (A, C), glial fibrillary acidic protein- GFAP (B, D), neuronal-specific nuclear protein-NeuN (E, F) and chondroitin sulphate-CS- 56 (G, H) immunocytochemistry. Framed area in A and B are enlarged correspondingly in C and D. The F and H represent high magnification of the callosal septa shown in E and G. Arrows point at thicker callosal septa, arrowheads at striations. Abbreviations: s – callosal septa, CC – corpus callosum, SCZ – subcallosal zone, V – cerebral ventricle, a – anterior, p – posterior, d – dorsal.

The typical structure of septa is already present in the youngest fetal group (18 PCW). The best overall picture of its structure is visible on low power magnifications of sections processed with GFAP immunocyitochemistry (Figure 1: B, D). On high power magnification (Figure 2A) it is clearly visible that callosal septa are the continuation of triangular crest-like protrusions of the subcallosal zone and the subcallosal-subventricular zone[9]. The intensive GFAP staining of septa is associated with immunoreactivity of small size vessels, presumably capillaries with perivascular GFAP staining belonging to astrocyte's elements, GFAP positive fibers of fine caliber, radial glia and scattered GFAP positive astrocytes (Figure 2A). The overlap and superposition of fibrillar, cellular and vascular elements contribute to the delineation of triangular shape of the ventral portion of the callosal septa, while borders of the cellular elements in septa are difficult to delineate clearly in the period of developmental peak (Figure 2A).

In order to see neuronal content we have examined sections with NeuN immuncytochemistry. These preparations show the presence of numerous neuronal nuclei distributed along the callosal septa with higher concentration in the ventral enlarged triangular portion (Figures 1: E arrows, F). The extracellular matrix content was examined on sections processed with chondroitin
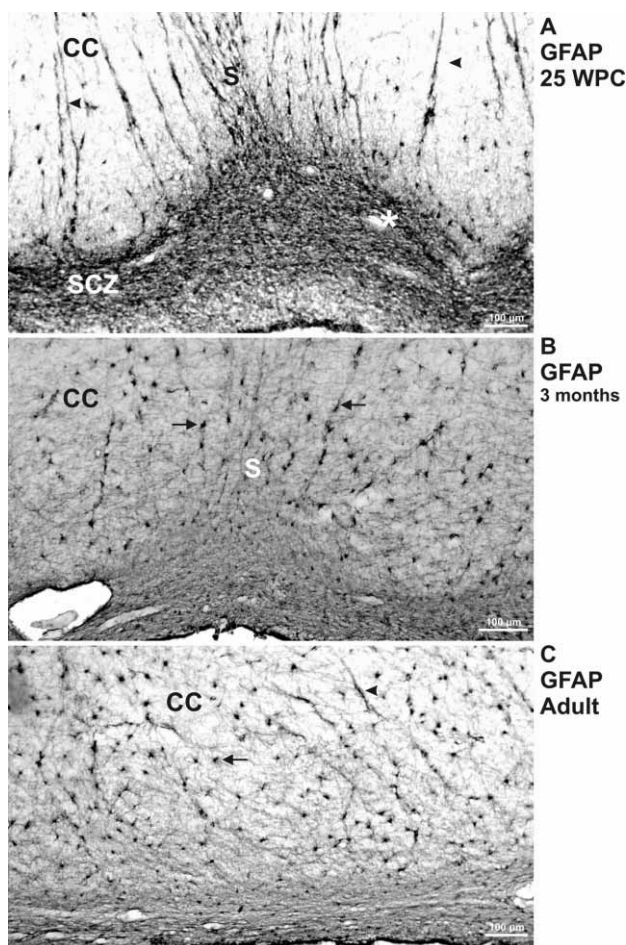
Fig. 2. The developmental changes of callosal septa in anterior corpus callosum, presented on mediosagittal sections of the human brain at age of 25 weeks post conception-WPC, 3 postnatal month and 42 years, immunocytochemicaly labeled with glial fibrillary acidic protein-GFAP. Arrowheads point at striations, asterisk at blood vessel. Abbreviations: s – callosal septa, CC – corpus callosum, SCZ – subcallosal zone, V – cerebral ventricle.

sulphate (CS-56) immunocyitochemistry (Figure 1: G, H). With this method we have seen higher content of CS-56 in septa during fetal and preterm phases. Thin, radially oriented striations are only as wide as one to three cell rows and are rather more evenly spaced on the anterior-posterior axis (Figure 1: G, H, arrowheads).

Developmental pattern

The prominent callosal septa are seen in the fetal and preterm brain until the 34th WPC. During this period the septa contain numerous NeuN positive nuclei, ventral triangular crest-like enlargement and a high content of ECM. The short and small septa disappear on low power magnification during the later stages. The large callosal septa which separate major callosal bundles seem to persist longer. In the brain of a three month postnatal infant only barely visible discrete stripes were present, with

sparsely in line spaced GFAP positive cells (Figure 2B, arrows), without chondroitin sulphate reactive ECM and NeuN positive nuclei. There is also a remarkable reduction of radially oriented thin striations in the postnatal corpus callosum. In the brain of an adult humans there are no more regularly arranged, radially oriented striations, instead, there is meshwork of randomly oriented GFAP positive fibrillar elements (Figure 2C arrowheads) predominantly associated with blood vessels and scattered GFAP positive astrocytes (Figure 2C arrows).

Regional differences

In the knee (genum), curved, anterior (frontal) part of the corpus callosum (Figure 1B, framed area), the callosal septa are more numerous and more regularly spaced showing radial orientation (Figure 1D arrow). In the corpus of the callosum, striations and septa are less numerous, while in the splenium number of septa and striations increase, but still in comparison within the genum, septa are not so prominent, they are more thinly and less numerous.

Discussion

We have described transient cellular structure, callosal septa, of variable size, which divide developing CC into irregular segments. To our knowledge these transient structures have not been specifically described in the literature. However, segmentation of the CC is visible in the illustrations of midsagittal sections of human[21,22] and monkey developing brain[23]. Bayer and Altman only named the subcallosal stratum as callosal glioepithelium in their illustrations of histological section from the Yakovlev collection[22]. The illustrated glioepithelium corresponds to the ventral continuation of the callosal septa, described previously as the subcallosal zone and the subcallosal-subventricular zone[9]. Our results clearly indicate that septa contain both neuronal and glial elements. The transient cells of the callosal septa and transient expression of particular ECM molecules in the septa indicate their possible role in morphogenesis of the CC. There are several lines of evidence which support the morphogenetic role of the callosal septa. First, the septa are formed as a scaffold for transverse growth of the callosal fibers. In this respect the septa would have a guidance role similar to the glial-neuronal sling during early CC development[6,8]. Second, the cells within the callosal septa may produce axonal guidance molecules necessary for active guidance of callosal axons during the midline growth towards the opposite hemisphere. This is supported by studies of mammalian brains showing that cells situated in the midline produce important attractant and repellant molecules such as members of Netrins, Slits or Semaphorins families of ligands and receptors[5,7,20,24]. However, the exact cellular sources of these molecules have never been convincingly identified *in situ*.

Third, the ECM content of septa may serve as a substrate for callosal axons growth along the ventral callosal

moiety during the midgestational period. The chondroitin-sulphate proteoglycan observed in the present study was shown to be a growth promoting molecule during the development of thalamocortical axons[25].

Furthermore, the period of developmental peak of the callosal septa corresponds to the most active period of growth of callosal fibers in man, that is between 18-32/34 WPC. The morphogenesis of the CC after 32/34 WPC is complicated by possible retraction of some callosal fibers[12] and disappearance of the waiting compartment[26]. However, in monkey brains the retraction of callosal axons seems to be a predominantly postnatal event[13]. Since the existence of remnants of callosal septa continues also after birth, they may also help in the process of retraction and withdrawal of callosal axons. Thick CC bundles on the roof of the lateral ventricle represent a structural barrier for migratory neurons. Thus, besides the role in growth of callosal fibers across midline, the septa may also serve for migration of the classes of neurons born later. The groups of neurons may use glial elements in the callosal septa to »climb« towards the cortex, therefore, the glial cells and fibers in the septa, observed in our study, may facilitate the migration of neurons originating from the subventricular zone. In addition, the callosal septa may play a role in providing topographically ordered commissural projection from one hemisphere to another. The corpus callosum of adult humans can be divided into »segments« which contain topogra-phically arranged callosal fibers for a given cortical area[27–32]. Thus, it may be assumed that the callosal septa described in the present study, help to maintain the topographical relationship of interhemispheric connection during the development of the CC. Indeed, the distribution of corpus callosum fibers in monkeys is established already during the prenatal period[2]. However, distances between the septa on the anterior-posterior axis of the CC are very irregular and their sizes are very variable which may not provide a precise topographical relationship. Although the corpus callosum develops in an anterior-posterior direction, it should be noted that at least in mice, newly added axons grow along the ventral aspect of the corpus callosum[33]. This experimental observation is in accordance with the enlargement of ventral edges of the callosal septa and may explain the abundance of cellular and ECM substrate along the ventral aspect of the corpus callosum.

Acknowledgements

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PROLAZNE STANIČNE STRUKTURE U RAZVITKU KORPUSA KALOZUMA ČOVJEKA

S A Ž E T A K

Corpus callosum (korpus kalozum, žuljevito tijelo) povezuje moždane hemisfere i predstavlja najvoluminozniji snop aksona u ljudskom mozgu. Tijekom razvitka komisuralna kalozalna vlakna polaze većinom od nezrelih piramidnih neurona moždane kore, rastu duž složenih putova, prelaze mediosagitalnu ravninu telencefalona, koristeći pri tom različite stanične supstrate u prolaznim fetalnim strukturama. U ovom radu analizirali smo strukture u korpusu kalozumu na postmortalnim histološkim rezovima ljudskog mozga od fetalne (18. tjedan nakon začeća) do odrasle dobi. Pronašli smo prolazne stanične strukture kalozalne prečage, između ventralnih snopova aksona, koje se spajaju sa subkalozalnom zonom i oblikuju žljebove u kojima su položeni kalozalni snopovi aksona. Prečage sadrže stanice i vlakna immunoreaktivna na glijalni fibrilarni protein, neurone i izvanstraničnu tvar bogatu hondroitin sulfatom. Tijekom ranog postnatalnog razdoblja kalozalne prečage postaju tanje i kraće, ne sadrže neurone i hondroitin sulfat, a ekspresija glijalnog fibrilarnog proteina bitno je smanjena. Razvojni vrhunac kalozalnih prečaga je u razdoblju od 18. do 34. tjedna od začeća, u vrijeme najintenzivnijeg rasta korpusa kalozuma u čovjeka, što ukazuje na razvojnu ulogu ovih struktura u njegovom rastu i oblikovanju. Ovi nalazi rasvjetljavaju dio složenih morfogenetskih zbivanja tijekom rasta korpusa kalozuma i daju neophodne parametre za daljnje proučavanje strukturne plastičnosti nakon perinatalnog oštećenja mozga.