Morphology and morphometry of the human embryonic brain: A three-dimensional analysis

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Abstract

The three-dimensional dynamics and morphology of the human embryonic brain have not been previously analyzed using modern imaging techniques. The morphogenesis of the cerebral vesicles and ventricles was analyzed using images derived from human embryo specimens from the Kyoto Collection, which were acquired with a magnetic resonance microscope equipped with a 2.35-T superconducting magnet. A total of 101 embryos between Carnegie stages (CS) 13 and 23, without apparent morphological damage or torsion in the brain ventricles and axes, were studied. To estimate the uneven development of the cerebral vesicles, the volumes of the whole embryo and brain, prosencephalon, mesencephalon, and rhombencephalon with their respective ventricles were measured using image analyzing Amira™ software. The brain volume, excluding the ventricles (brain tissue), was 1.15 ± 0.43 mm³ (mean ± SD) at CS13 and increased exponentially to 189.10 ± 36.91 mm³ at CS23, a 164.4-fold increase, which is consistent with the observed morphological changes. The mean volume of the prosencephalon was 0.26 ± 0.15 mm³ at CS13. The volume increased exponentially until CS23, when it reached 110.99 ± 27.58 mm³. The mean volumes of the mesencephalon and rhombencephalon were 0.20 ± 0.07 mm³ and 0.69 ± 0.23 mm³ at CS13, respectively; the volumes reached 21.86 ± 3.30 mm³ and 56.45 ± 7.64 mm³ at CS23, respectively. The ratio of the cerebellum to the rhombencephalon was approximately 7.2% at CS20, and increased to 12.8% at CS23. The ratio of the volume of the cerebral vesicles to that of the whole embryo remained nearly constant between CS15 and CS23 (11.6–15.5%). The non-uniform thickness of the brain tissue during development, which may indicate the differentiation of the brain, was visualized with surface color mapping by thickness. At CS23, the basal regions of the prosencephalon and rhombencephalon were thicker than the corresponding dorsal regions. The brain was further studied by the serial digital subtraction of layers of tissue from both the external and internal surfaces to visualize the core region (COR) of the thickening brain tissue. The COR, associated with the development of nuclei, became apparent after CS16; this was particularly visible in the prosencephalon. The anatomical positions of the COR were mostly consistent with the formation of the basal ganglia, thalamus, and pyramidal tract. This was confirmed through comparisons with serial histological sections of the human embryonic brain. The approach used in this study may be suitable as a convenient alternative method for estimating the development and differentiation of the neural ganglia and tracts. These findings contribute to a better understanding of brain and cerebral ventricle development.

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Introduction

Originating from a simple neural tube, the brain becomes an elaborate structure through a series of differentiation processes (O’Rahilly and Müller, 2006; Bayer and Altman, 2008; Huang et al., 2009). The three brain vesicles that form at the cranial end of the neural tube differentiate to form the prosencephalon, mesencephalon, and rhombencephalon at Carnegie stage (CS) 13 (O’Rahilly and Müller, 1987). CS is a standardized system of 23 stages used to provide a unified developmental chronology of the human embryo. The stages are delineated through the development of external and internal structures, not by size or the number of days of development. The brain achieves its definitive organization after CS15 with the growth of the telencephalon. The diencephalon becomes enclosed between the cerebral hemispheres on both sides.

Classically, three-dimensional (3D) developmental anatomy was analyzed with serial histological sections and visualized with 3D modeling...
and/or illustrations. Those methods were laborious and inaccurate in the translation of two-dimensional histology into a 3D object. Recent advances in magnetic resonance (MR) imaging, computed tomography (CT), and 3D sonography have provided 3D digital information, which has been applied to clinical diagnosis. In the field of fetal medicine, 3D images of the fetal brain acquired with MR imaging during the second trimester have been studied (Kinoshi et al., 2001; Huang et al., 2009); however, 3D imaging during the embryonic period proper (the first 2 months of the first trimester) have not been fully analyzed. In the present study, the morphogenesis of the human embryonic brain was analyzed via 3D reconstructions from MR microscopic data. The data provided demonstrates the dramatic growth of the human brain during the embryonic period in each CS.

**Materials and methods**

*Human embryo specimens*

Approximately 44,000 human embryos comprising the Kyoto Collection are stored at the Congenital Anomaly Research Center of Kyoto University (Nishimura et al., 1968; Shiota, 1991; Yamada et al., 2006; Shiraishi et al., 2013). In most of these cases, pregnancy was terminated during the first trimester for socioeconomic reasons under the Maternity Protection Law of Japan. Approximately 20% of the embryos were not damaged and were well preserved. In the laboratory, aborted embryos were measured, examined, and staged using the criteria provided by O'Rahilly and Müller (1987). Approximately 1200 well-preserved human embryos found by two of the authors (C.U. and S.Y.) to be normal on gross examination and between CS13 and CS23 were selected for MR microscopic imaging. The conditions used to acquire the MR images of the embryos have been previously described elsewhere (Matsuda et al., 2003, 2007; Yamada et al., 2006; Shiota et al., 2007). Briefly, the MR images of the embryos were acquired using a super-parallel MR microscope developed with a 2.35 T horizontal bore (40 cm) superconducting magnet (Matsuda et al., 2007). The pulse sequences used for the image acquisition were T1-weighted spin echo sequences with 100 ms repetition times and 10–16 ms echo times. The image matrix was 128 × 128 × 256 and the size of the voxel varied from (40 μm)³ to (150 μm)³. Because the number of signal accumulations was 16 or 24, the total data-acquisition time was 7.3 or 10.9 h. As shown in the previous paper (Matsuda et al., 2007), the image intensity of the T1-weighted images of the human embryos has a close correlation with that of Nissl staining sections.

For the present study, 101 samples at different CS between CS13 and CS23 (9 or 10 samples for each stage, except CS13, for which there were 5 samples) were selected from the 1200 MR image datasets for 3D reconstruction and morphometric analysis. The selected embryos were re-examined by two authors (T.N. and T.T.) based on previously described criteria (Nakashima et al., 2012). The samples with apparent deformity and brain shrinkage were excluded from the analysis because prolonged fixation is known to cause MRI artifacts and tissue shrinkage due to dehydration (van Duijn et al., 2011).

**Three-dimensional reconstruction and morphometric analysis**

Three-dimensional MR image datasets for each embryo were resectioned as sequential 2D images digitally with ImageJ64™ (ver. 1.44, National Institutes of Health, Bethesda, Maryland, United States) and saved as Analyze file formats (.hdr, .img). On MR imaging, brain tissue showed layered structures with high intensity signals. It was difficult to make precise distinctions between histological structures within the brain tissue, though the borders between brain tissues and the surrounding tissues, such as the subarachnoid spaces, ventricles, and mesenchymal tissues, were clear (data not shown).

The brains and ventricles were segmented for 3D reconstruction using FSL View of FMRIB Software Library™ (ver. 4.1.9, Analysis Group, FMRIB, Oxford, UK). Three-dimensional morphometry of the brain was computationally reconstructed with Amira™ software (ver. 5.4.0, Visage Imaging, Berlin, Germany).

The regional non-uniform thickness of the brain tissue was visualized using the following two filter modules of the Amira™ software program: 1) surface thickness (the thickness of the brain was visualized on the surface with a color scale) and 2) extraction of the “core region.” Layers of tissue were digitally subtracted from the brain tissue by equal amounts from both the external and internal surfaces. The remainder was visualized as the core region (COR) of the thickening brain tissue. The dorso-lateral part of the telencephalon (cortex) was used as a reference to determine the number of layers needed to be subtracted to isolate the COR in the respective samples.

The volumes of the brain and whole embryo were calculated using OsiriX™ software (ver. 4.0, Pixmeo SARL, Geneva, Switzerland). The brain vesicles were divided into three regions according to anatomic landmarks. The supramammillary recess and posterior commissure were used to define the prosencephalon and mesencephalon, the isthmic recess and the isthmic groove to define the mesencephalon and rhombencephalon, and the level of the C1 vertebra to define the separation between the rhombencephalon and spinal cord (Nakashima et al., 2012). The cerebellum was segmented using the Amira software program after visualization of the COR. The anatomical references used were the isthmic groove and the roof of the fourth ventricle.

This study was approved by the Committee of Medical Ethics of Kyoto University Graduate School of Medicine, Kyoto, Japan (E986).

**Results**

*Morphogenesis of the reconstructed brain*

All 101 brains between CS13 and CS23 were reconstructed for morphological and morphometric analysis. The 3D reconstruction allowed us to make precise gross observations (Fig. 1A, Supplementary File 1 in Shiraishi et al., submitted for publication). The three brain vesicles, formed at the cranial end of the neural tube, differentiated to form the prosencephalon, mesencephalon, and rhombencephalon at CS14. The telencephalon differentiated from the prosencephalon after CS15, and cerebral hemispheres became apparent on both sides of the diencephalon at CS17. The cerebral hemispheres grew in a pattern similar to that of a ram horn, with an arch directed backward and spirally outward (known as the rotation of the hemispheres) until CS23. Changes in internal morphology, including the formation of the ventricular system, were also recognizable (Fig. 1B). During the dynamic embryonic differentiation of the prosencephalon, the lateral ventricles (LVs) and the third ventricle formed, and the fourth ventricle developed in the rhombencephalon.

*Morphometry of the reconstructed brain*

The brain tissue volume and ventricular volume between CS13 and CS23 were calculated for all 101 reconstructions samples. The brain volume excluding the ventricles (brain tissue) was 1.15 ± 0.43 mm³ at CS13 and increased exponentially to 189.10 ± 36.91 mm³ at CS23 (Fig. 2). The brain tissue volume increased 164.4-fold, which is consistent with the observed morphological changes (Fig. 1A and B).

To assess brain growth during the embryonic period, the volume of brain tissue was compared to the whole embryo volume. The whole embryo volume was 12.73 ± 3.63 mm³ at CS13 and exponentially increased to 1453.84 ± 418.05 mm³ at CS23. The ratio of brain tissue volume to embryo volume was 9.0% at CS13 and increased to 13.1% at CS15. The ratio remained within a narrow range after CS15, between 11.6% (CS17) and 15.5% (CS22). The brain tissue volume expanded 34.7-fold, compared with a 34.9-fold expansion of the whole embryo volume, between CS15 and CS23. This data indicates that the growth rate of brain tissue and that of the whole embryo is comparable between CS15 and CS23.
The tissue volume of the main three vesicles was also calculated. Though the diencephalon and telencephalon differentiated after CS15, the border of these two vesicles was not clearly discernible using the present MR data up to CS23. The tissue volume of the three brain vesicles grew exponentially. The brain tissue volumes of the prosencephalon, mesencephalon, and rhombencephalon were 0.26 ± 0.15 mm³, 0.20 ± 0.065 mm³, and 0.69 ± 0.23 mm³ at CS13, respectively. The volume increased exponentially, and reached 110.99 ± 27.58 mm³, 21.86 ± 3.30 mm³, and 56.45 ± 7.64 mm³ at CS23, respectively (Fig. 3). The tissue volume of the rhombencephalon was higher than that of the prosencephalon between CS13 and CS19. However, the tissue volume of the prosencephalon was higher than that of the rhombencephalon after CS20 as the volume of the prosencephalon increased faster than that of the rhombencephalon.

The brain tissue volume was compared with the cavity volume. The internal cavities of the brain, namely the brain ventricles, were prominent especially in the early embryonic stages. The brain ventricle volume was 0.94 ± 0.38 mm³ at CS13 and increased to 97.25 ± 28.29 mm³ at CS23 (Fig. 4A). The volume of the cavities was greater than that of the brain tissue between CS14 and CS18. The ratio of the whole brain cavity volume to tissue volume reached a maximum at CS17 (1.46). After CS17, the volume of brain tissue was greater than that of the prosencephalon between CS13 and CS19. However, the tissue volume of the prosencephalon was higher than that of the rhombencephalon after CS20 as the volume of the prosencephalon increased faster than that of the rhombencephalon.
The non-uniform thickness of brain tissue during development

As the brain develops, the thickness of brain tissue changes and contributes to the formation of this complicated organ. We visualized these dynamic changes in thickness on the brain surface using a color scale (Fig. 5, Supplementary File 2 in Shiraishi et al., submitted for publication). The brain was relatively uniform in thickness between CS13 and CS16. However, non-uniformity in the thickness of the brain tissue was distinct after CS17. The ventricular eminences were detectable after CS17. The base of the telencephalon (basal ganglia) and the rhombencephalon, along with the primordial cerebellum become thickened, while the alar parts of the telencephalon, mesencephalon, and rhombencephalon remained thin.

The filter module use to serially subtract brain tissue from the external and internal surfaces of the developing brain enabled 3D visualization of the core regions of the thickening brain tissues resulting from brain differentiation and growth (Fig. 6). The COR correlated to the development of the nuclei and tracts and became apparent after CS17. This thickening was first seen in the basal parts of the rhombencephalon, cranial nerve nuclei, and the lateral and medial ventricular eminences in the prosencephalon. The anatomical positions of the COR were mostly consistent with nuclei such as the basal ganglia, thalamus, hypothalamus, pyramidal tract, and cranial nerve nuclei. The differentiation of the cerebellum was visualized as well. This was confirmed through a comparison with our own serial histological sections and an atlas of the human embryonic brain (Gasser et al., 2014; Bayer and Altman, 2008) (see Supplementary File 1 in Shiraishi et al., submitted for publication). A right–left difference regarding the increasing thickness of the brain tissue was not detectable in the present study.

The differentiation of the rhombencephalon is unique and complicated, and morphological descriptions are limited, especially with regard to the internal view and thickening of the brain tissue. The brain cavity of the rhombencephalon, the eventual fourth ventricle, did not grow exponentially and reached its maximum volume at CS19 (Fig. 4B). Nonuniformity in the thickness of the rhombencephalon tissue was visualized as external and internal cerebellar swelling, detectable after CS18 (Fig. 7, Supplementary File 3 in Shiraishi et al., submitted for publication). The ratio of the cerebellum to the rhombencephalon was approximately 7.2% at CS20, and increased to 12.8% at CS23 (Fig. 3).

Discussion

The dynamics of embryonic brain development have not been fully described. Most of the limited data available describe the initiation of fetal brain growth (Grenell and Scammon, 1943; Dunn, 1921). Jenkins (1921) measured the relative weight and volume of the component parts of the brain in 10 samples at different stages of development, including three samples from the embryonic period. With regard to the embryonic period proper, Desmond and O’Rahilly (1981) measured the major axes of the three embryonic brain vesicles and showed that the rates of growth of all three embryonic brain vesicles were much greater than the growth of the corresponding vesicles during the fetal period. Levitan and Desmond (2009) measured the areas of median sections two dimensionally to describe the growth of the three primary brain vesicles and to determine the change in the ratio of tissue area to cavity area. These studies were not able to accurately reconstruct the growth of the brain, region by region.

In the present study, we created a precise 3D reconstruction of the human embryonic brain with MR imaging data from 101 samples. We were able to accurately characterize the morphology and anatomy throughout the different stages of human embryonic brain development, improving our understanding of this highly ordered process.

Three-dimensional morphometry was possible after sectioning the brain. We were able to measure and analyze these digital sections to study the morphology and volume of the whole embryo and brain and the prosencephalon, mesencephalon, and rhombencephalon, with their respective ventricles. The present data may be applied as a standard for each stage, having been derived from a statistically adequate sample size.

The ratio of embryonic brain tissue volume to the whole embryo volume increased from CS13 to CS15, and then remained nearly
constant between CS15 and CS23. Levitan and Desmond (2009) previously noted that an increase in the volumes of the brain cavities is a feature of the embryonic period. The cavity volume was relatively large, but there was no exponential increase in cavity volume, despite the complicated changes that the cavity underwent in the late embryonic stages in our study. The brain demonstrates conspicuous growth during the embryonic periods, which in a large part is the result of an expansion of the brain cavity. This increase in cavity volume may result in an overestimate of the growth rate of the brain. During the period of organogenesis, almost all organs other than the brain simultaneously develop at maximum speed. The present data indicate that the growth rate of the brain tissue alone is not as rapid during organogenesis, except perhaps in the earlier stages between CS13 and CS15.

Morphometry of the three major divisions of the embryonic brain clarified the features of the individual brain vesicles. The present study showed that the morphology of both the brain tissue and cavity of the rhombencephalon was affected by the development of the pons and medulla of the brain stem and the cerebellum according to their respective growth time lines. Previous studies have suggested that morphometric changes of the rhombencephalon differ from the other vesicles (Desmond and O'Rahilly, 1981; Levitan and Desmond, 2009). In our study, growth of the brain stem was apparent until CS17, prior to detectable growth of the cerebellum. External and internal cerebellar swellings were detectable after CS18. This complicated morphogenesis is responsible for the morphometric changes. The volume of the tissue and cavity increased in a non-exponential fashion and cavity volume reached a maximum at CS19.

In contrast to previous studies (Desmond and O’Rahilly, 1981), the growth rate of the rhombencephalon exceeded that of the prosencephalon until CS19. Morphological analysis in the present study revealed that the ventral part of the rhombencephalon thickened, corresponding to growth of the brain stem and neural tracts. In contrast, the prosencephalon appeared larger with the emergence of the cerebral hemispheres on both sides, but the tissues of these structures remained thin during the embryonic period. After CS20, the growth of the prosencephalon becomes much greater than that of the other 2 sections, as the cerebral hemispheres grow rapidly, contributing to the exponential growth of the brain observed during the fetal period. The rate of growth of the prosencephalon continues to be greater than that of the rest of the brain.
brain throughout the fetal period and into postnatal development and adulthood (Dunn, 1921; Jenkins, 1921; Grenell and Scammon, 1943).

Embryonic development is characterized by dynamic and obvious changes in external appearance in accordance with internal organogenesis. To visualize the non-uniform regional thickness of the tissues of the embryonic brain during development we employed surface color mapping by thickness and extraction of the COR of the thickened brain tissue by the digital subtraction of layers of tissue. Brain tissue may grow in two directions. Eccentric growth occurs without thickening of the brain tissue, while concentric growth results in brain tissue thickening. It is well known that flexion/extension occurs during the formation of the highly structured brain during the embryonic period. Surface mapping by thickness in the present study demonstrated the correlation between the dynamic structure (flexion/extension) and the direction of tissue growth (thickness). The tissue became thicker in the regions of flexion while it remained thin in the regions of extension. Surface mapping enabled us to visualize both surface morphology and internal thickness at the same time.

**Fig. 6.** The core region (COR) of the thickening brain tissue. The amount of tissue digitally subtracted to isolate the COR was determined using the dorso-lateral region of the telencephalon as a reference. The thickness subtracted was 0.28 mm for the CS17 brain, 0.4 mm for the CS20 brain, and 0.95 mm for the CS23 brain. Ce: cerebellum.
The brain core was isolated by digital subtraction of layers of tissue from both the external and internal surfaces of the developing brain in the present study. This allowed us to visualize the COR of the thickening brain where concentric growth had occurred. The COR appeared to correspond to the growth of cerebral nuclei, cranial nerve nuclei, and pyramidal tracts when the anatomical positions of growth were compared with our own histological sections and an atlas of the embryonic brain (Gasser et al., 2014; Bayer and Altman, 2008) (Supplemental Fig. 1). The COR, as defined in the present study, may be valuable as an anatomical indicator of the development and differentiation of the nuclei and tracts in the brain. Histological study and/or brain imaging study using diffusion tensor image (DTI) techniques with fresh tissues of human embryos are necessary to precisely reveal the development of the neural ganglia and tracts. Previous fetal brain imaging studies using diffusion tensor image have been performed in the second trimester (Huang et al., 2009). Although fresh tissues from human embryos are difficult to obtain, our methods may be suitable as a convenient alternative for studying the development and differentiation of the neural ganglia and tracts.

Recent morphological approaches to the study of human embryonic development have become multi-directional with the aid of high-resolution digital imaging. The development of the embryonic brain has been previously examined using histology-based methods. Multiple histological studies have been published, which describe the precise

Fig. 7. Representative 3D images of the rhombencephalon with surface color mapping at CS17, 20, and 23. The roof of the rhombencephalon has been removed to reveal the inside tissues of the fourth ventricle, demonstrating the internal thickenings in the pons and medulla (brain stem) and cerebellar regions. *, Internal cerebellar swelling; +, rhombic lip. Closer view of the rhombencephalon is shown in Supplementary File 3 in Shiraishi et al. (submitted for publication).
local developmental anatomy. However, precise knowledge regarding the 3D volumes and shapes of structures within the developing human brain has been lacking. Gasser et al. (2014) has “rebirthed” a huge amount of histological data with an accessible and comprehensive database (http://virtualhumanembryo.lsuhsc.edu/). The database consists of all levels of each sectional image shown with 3D reconstruction. Such embryo visualization programs provide valuable image data sets of human embryos for the education of students and obstetricians, as well as for the study of human development. The Human Developmental Studies Network (Kerwin et al., 2010, HuDSen; http://www.hudsen.org/) is a unique database that aims to provide a forum for researchers in human developmental biology and related fields such as experimental developmental molecular and cellular biology. This network allows these researchers to establish links and exchange information. The integration and analysis of gene expression data from various stages of human development will enable a comparative analysis between human and mouse spatio-temporal gene expression data (HuDSen human gene expression spatial database) in the future.

Clinically, sonography is convenient and low-risk compared to other imaging methods such as MRI and CT. Three-dimensional sonography, performed with high-frequency transvaginal transducers, has expanded the depth of inquiry and allowed 3D sonoembryology (Blaas and Eik-Nes, 2009; Pooh et al., 2011). These sophisticated sonoembryographic imaging techniques allow the definition of in vivo anatomy including visualization of the embryonic blood circulation, cerebrospinal fluid flow, and other dynamic features that cannot be characterized in fixed specimens (Pooh, 2012a, 2012b; Pooh and Kurjak, 2011). In addition, 3D sonoembryology provides a basis for the assessment of anomalies as well as normal human development, and will contribute to more accurate prenatal diagnoses (Blaas, 2014).

Most MR imaging-based anatomical studies like ours have been limited by the use of expired embryos stored in formalin. Research using clinical data from sonography will be valuable as any artifacts, due to prolonged storage in formalin, would be eliminated (van Duijn et al., 2011). Our study does have the advantage of providing a convenient 3D reconstruction and quantitative morphometric assessment of the embryonic brain, especially regarding the dynamic changes that occur during brain development. The present study provides information complementary to information from histology, sonoembryology, and experimental biology. This greatly expands our understanding of brain growth and provides unique data for future studies.

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