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Evidence for novel age-dependent network structures as a putative primo vascular network in the dura mater of the rat brain

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Abstract

With chromium-hematoxylin staining, we found evidence for the existence of novel age-dependent network structures in the dura mater of rat brains. Under stereomicroscopy, we noticed that chromium-hematoxylin-stained threadlike structures, which were barely observable in 1-week-old rats, were networked in specific areas of the brain, for example, the lateral lobes and the cerebella, in 4-week-old rats. In 7-week-old rats, those structures were found to have become larger and better networked. With phase contrast microscopy, we found that in 1-week-old rats, chromium-hematoxylin-stained granules were scattered in the same areas of the brain in which the network structures would later be observed in the 4- and 7-week-old rats. Such age-dependent network structures were examined by using optical and transmission electron microscopy, and the following results were obtained. The scattered granules fused into networks with increasing age. Cross-sections of the age-dependent network structures demonstrated heavily-stained basophilic substructures. Transmission electron microscopy revealed the basophilic substructures to be clusters with high electron densities consisting of nanosized particles. We report these data as evidence for the existence of age-dependent network structures in the dura mater, we discuss their putative functions of age-dependent network structures beyond the general concept of the dura mater as a supporting matrix.

Key Words: *nerve regeneration; dura mater, chromium-hematoxylin staining; fascia; primo vascular system; brain; hormone; neural regeneration*

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Introduction

Does the brain also function as a hormone-containing organ like an adrenal gland? Such an idea seems unreasonable in modern neuroscience, and the idea of a neurohormonal system in the brain would have been impossible to validate without the chromium-hematoxylin (Cr-Hx) staining method (Bargmann, 1949). Cr-Hx staining of the brain has shown that the pituitary contains and releases hormones (Dawson, 1953; Peterson, 1966). In previous works, our team applied Cr-Hx staining to visualize new anatomical structures in the venous sinuses of the rat brain (Lee et al., 2012). Those novel threadlike and node-like anatomical structures, called primo vessels and primo nodes, respectively, were strongly stained by Cr-Hx, and their existence suggests that a specific network system was floating inside the brain's venous sinuses.

Ironically, this new finding in the brain is consistent with an existing working hypothesis, namely, that the acupuncture meridian system, a key channel network in Oriental medicine, is a real anatomical system, called the primo vascular system (Kim, 1962, 1963a, b, 1965a, b, 2013; Lee et

al., 2005, 2013a, b, c, d, e, 2014; Soh, 2009; Han et al., 2010, 2013; Lee, 2012; Bae et al., 2013; Huh et al., 2013; Islam et al., 2013; Kang, 2013; Moon et al., 2013; Ping et al., 2013; Stefanov et al., 2013; Bae and Soh, 2014; Jung et al., 2014; Nam et al., 2014; Park et al., 2014a, b), with a high affinity for chromium. However, because some hormones such as adrenaline and noradrenaline are known to show a strong affinity for chromium salt (Nordenstam and Adams-Ray, 1957; Pinheiro et al., 2014), we hypothesized that one of the main functions of the primo vascular system is to contain and transport hormones in the same way that neurohormonal tissue in the brain does. Very recently, using the same dye, Cr-Hx, we found novel threadlike anatomical structures floating and embedded in the epicardium of a rat's heart (Lee et al., 2013d). The epicardium of the heart is a kind of connective tissue that makes up the entire fascia system in a living organism (Bai et al., 2011; Finando and Finando, 2011; Findley et al., 2012; Cheng, 2014). Interestingly, such connective tissues in the meninges are known to protect brain tissues in the same way as the epicardium protects the heart. From these viewpoints and with these data, we hypothesized that the meninges in the brain might have specific hidden anatomical structures with characteristics that are the same as those in previously found novel anatomical structures (Kim, 1962, 1963a, b, 1965a, b, 2013; Lee et al., 2005, 2013a, b, c, d, e, 2014; Soh, 2009; Han et al., 2010, 2013; Lee, 2012; Bae et al., 2013; Huh et al., 2013; Islam et al., 2013; Kang, 2013; Moon et al., 2013; Ping et al., 2013; Stefanov et al., 2013; Bae and Soh, 2014; Jung et al., 2014; Nam et al., 2014; Park et al., 2014a, b).

To verify our hypothesis, we applied Cr-Hx staining to the meninges in rat brains and examined specimens of the stained areas under microscope.

Materials and Methods

Animals

Male Wistar rats aged 1–7 weeks (n = 3 for 1 week, 1 for 2 weeks, 4 for 4 weeks, and 2 for 7 weeks) provided by Samtako Bio Korea, Bio Korea, Gyunggi-Do, Korea, were housed in a room that was temperature controlled at 24–25°C and light-controlled with a 12-hour light/dark cycle. They were provided water and commercial rat chow *ad libitum* and were acclimatized for 1 week before the experiments. These experiments were carried out in accordance with the guidelines of the Laboratory Animal Care Advisory Committee of the Korea Advanced Institute of Science and Technology (KAIST approval No. KA2011-13). The rats were anesthetized by using an intramuscular injection of a combination of ketamine (45 mg/kg) and lompun (5 mg/kg) into the right hind femoral limb. The procedure for preparing the chromium-hematoxylin solution (Cr-Hx) was reported in our previous work (Lee et al., 2012).

Surgical and observation procedures

To expose the meninges of the rat brains, under deep anesthesia, we injected more than enough urethane into the femoral vein of the rats to induce immediate death and avoid unnecessary pain. Immediately after that, we decapitated the rats. By using a rongeur, we removed the skull and exposed the dura mater of the brain's meninges. Under a stereomicroscope (SZ61, Olympus, Tokyo, Japan), we applied Cr-Hx drop by drop to the exposed dura mater of the rat brain. Immediately after that, we washed the specimen with phosphate buffered saline (pH 7.4) twice or three times. We performed this sequence of processes until the networks could be visualized under a stereomicroscope.

Microscopic examination

All visualized spotted and networked structures found at each age (1, 4, and 7 weeks) were first examined by using a stereomicroscope (SZ61) with a CCD camera (eXcope X3, DIX, Daejeon, Korea). Then, the isolated specimens were completely stained with 4′,6-diamidino-2-phenylindole (DAPI) and examined using an fluorescence optical microscope (BX 53, Olympus). Especially the size and the number of granules of the examined specimens were measured by phase contrast microscopy. After the entire specimen had been fixed in 10% neutral buffered formalin, it was cut into 10-μm cross sections for hematoxylin-eosin staining. For transmission electron microscopy examinations, the specimens were fixed in 2.5% paraformaldehyde and 2.5% glutaraldehyde in a neutral 0.1 M phosphate buffer solution for 1 hour. After post-fixation for 1 hour in 1% (w/v) osmic acid dissolved in PBS, the specimens were dehydrated in graded ethanol and embedded in Epon812 (EMS, Fort Washington, PA, USA). Semi-thin sections of 1 μm thickness stained with 1% toluidine blue were observed under an optical microscope (BX 53, Olympus) with a CCD camera (eXcope X3. DIX). Based on the observations of the semi-thin sections, we cut ultrathin sections, mounted them on nickel grids, and double stained them with uranyl acetate, followed by staining with lead citrate. The sections were examined with a Technai G2 Spirit transmission electron microscope (FEI, USA).

Results

Visualization of age-dependent network structures (ADNS) under stereomicroscopy

As shown in **Table 1**, in the dura mater of the rat brain, we found novel network structures that varied with respect to their morphologies (granular forms or straight lines) and their distributions according to age (1, 2, 4, and 7 weeks). The table demonstrates that, with aging, the novel network structures gradually changed from granular forms to straight lines. Because the diameters of those structures also increased with age, we called them ADNS. The data are summarized in illustration form on the left side of **Figure 1**. This illustration shows the location of the ADNSs and their distribution. In the stereoscopic images shown in **Figure 1** for each age (1, 4, and 7 weeks), the ADNSs were barely visible in the brain of a 1-week-old rat; however, they were distinctly visible in the brains of the 4- and the 7-week-old rats. **Table 1** and **Figure 1** also show that the diameter of the ADNSs increased with age from 12.7 μm at 1 week to 17.7 μm at 7 weeks.

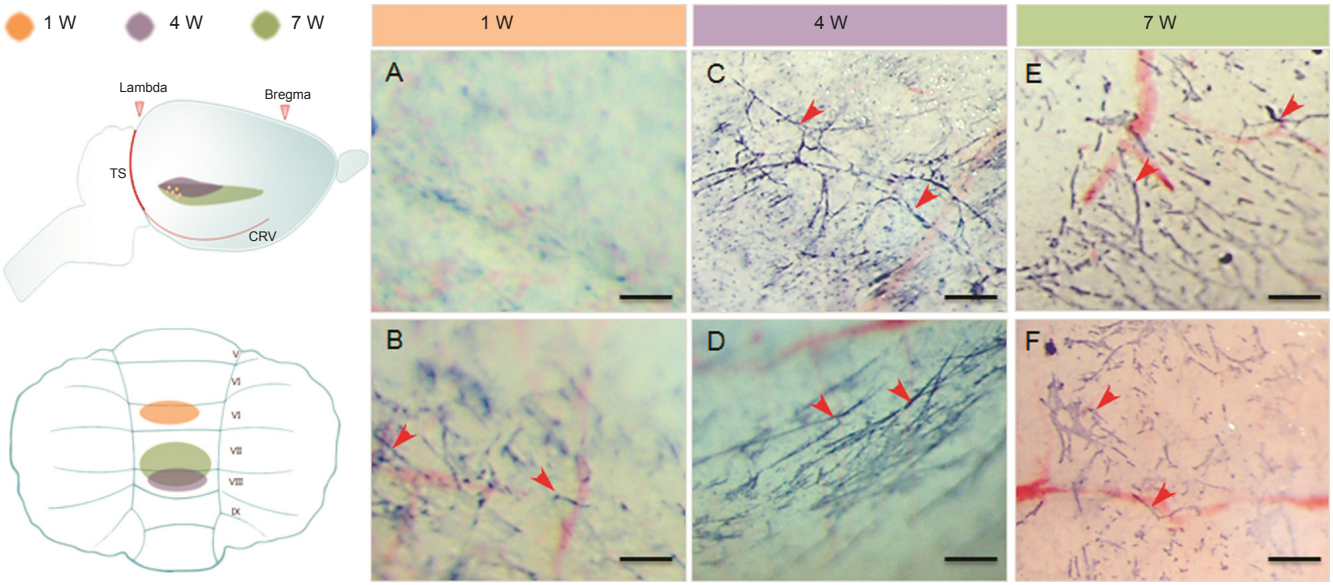

Figure 1 Illustrations and corresponding stereomicroscopic images of the age-dependent network structures (ADNSs) visualized (arrowheads) in the dura mater of the rat brain at 1, 4, and 7 weeks of age.
In the illustration on the left, A, C, and E show that the ADNSs are localized in the dura mater on the left and right temporal lobes, whereas B, D, and F show that the ADNSs also exist in the dura mater of the cerebellar vermis. TS: Transverse sinus; CEV: caudal rhinal vein. Under stereomicroscopy, the ADNSs are barely visible in the temporal lobes in the brain of a 1-week-old rat (A); however, some partial ADNSs (arrows) can be seen in B. C and D show the ADNSs in the dura mater of the 4-week-old rat brain. E and F show that the area in which the ADNSs exist has increased in the dura mater of the 7-week-old rat brain. All scale bars are 130 μm. Two points are noticeable: 1) the sizes of the ADNSs increase with age, and 2) the 1-week-old rat only shows scattered granules in the temporal lobes. W: Week(s).

Table 1 Experimental data to demonstrate the location, maximum thickness and number of granules for the ADNSs visualized in the dura maters of the brains of rats

Subject number	Age (week)	Location	Maximum size (μm)	Granule
1		x		+++
2	1	c	3.5–6.1	+++
3		c		+++
4	2	abc	10.7	+++
5		abc		++
6	4	abc	12.7–15.6	++
7		abc		+
8		abc		++
9	7	abc	17.7	++
10		abc		+

The maximum thickness indicates how large the ADNSs are visualized in diameter. a, b and c indicate the left and the right temporal lobes and the cerebellar vermis, respectively. The locations were determined using stereomicroscopy. The size and the number of granules were measured by phase contrast microscopy. We added the data for the ADNSs in 2-week-old rats (not shown in the figures). In location, "x" means ADNS is hardly visualized. In granules, the numbers of '+' suggest relative numbers of granules visualized in the same age. ADNS: Age-dependent network structures.

Hypothetical and real morphological changes in the ADNS with age

Based on the data, we hypothesized that the ADNSs develop from granular forms to threadlike channels with time. This hypothesis is illustrated in **Figure 2**, where each age (1, 4, and 7 weeks) is matched with a representative image. The image for the 1-week-old rat shows granules that seem to be strung along as on a thread. Some scattered granules also exist between the threads formed by the majority of the granules. At 4 weeks, the granules started to fuse into rod-shaped structures. Noticeably, some granules have fused into rod-shaped structures, while others were still separated and lay around those that had fused. At 7 weeks, clearly rod-shaped structures with distinct nuclei had formed. Noticeably, almost no scattered granules were seen around the fused structures at 7 weeks.

Characterization of the ADNS

To characterize the ADNS, we performed optical and electron microscopy measurements on specimens taken from a 4-week-old rat. As the inset of **Figure 3A** shown, the image of the ADNS stained with hematoxylin-eosin shows that it consisted of basophilic materials above the border of the cell layer. These basophilic materials in the hematoxylin-eosin image of the ADNS correspond to clusters with high electron densities in the transmission electron microscopy image in **Figure 3A**. Even though the hematoxylin-eoxin image of the ADNS seems to show one basophilic mass, its transmission electron microscopy image demonstrates that the mass consisted of several clusters. A magnified view of the clusters shows that they were made of nano-sized particles, while some particles formed vesicles and others were scattered among them, and each cluster of nano-sized particles was surrounded by a membrane. Noticeably, the ADNSs were enclosed by cytoplasm around the nucleus of a fibroblast.

Discussion

The data obtained in this study indicate that a novel network system, which can be stained using Cr-Hx, develops in the dura mater of the rat brain over time (age). This observation raises a simple question. Why until recently was this structure not recognized in brain science? The putative answer to this question may derive from the distribution pattern for this novel structure. These newly found structures change from granules to threadlike networks in an age-dependent manner. Therefore, if the entire age-dependent change process in the brain is not observed, the structure in the dura mater may not be recognized as a novel anatomical network.

Moreover, two additional fundamental questions still need to be answered before these novel structures can be established as an ADNS: Why were granules detected in the early stage of brain development? Why did they seem to fuse into threadlike structures with age? These questions are too fundamental to be answered with our data at present. However, even though we do not yet have convincing answers to these questions, our data suggest that the early emergence of scattered granules in the dura mater of the brain is closely related with the age-dependent formation of the ADNSs.

To visualize the ADNSs, we applied Cr-Hx staining to the dura mater of the brain. Since Bargmann's observation (Bargmann, 1949), slightly-modified methods have been used to visualize hormone-containing brain tissues, such as the hypothalamus or the pituitary (Sloper, 1955). Our stereomicroscopy images of the ADNSs in the dura mater of rat brains demonstrate that the ADNSs can be heavily stained by Cr-Hx, and our optical microscopy images indicate that the granules in the ADNSs can also be heavily stained by Cr-Hx. Moreover, the electron microscopy data on the ADNSs converge to one common fact: the existence of many granules with high electron densities. For further study, we suggest three important topics: (1) cultivation of the granules to characterize the interrelationship between the granules and the newly-found cellular structure, the ADNS; (2) analysis of the biochemical components in the ADNSs to determine their biological functions; and (3) elucidation of the age-dependent nature of the ADNSs, *i.e.*, the morphological and functional changes of the ADNSs during normal aging or dementia.

Now, studies addressing the patterns of the ADNSs in the entirety of a living body would be meaningful. For this, we have added a phase-contrast movie file to show how the ADNS is connected to the fine connective fiber that makes up the dura mater of the brain (supplemental data). All our data demonstrate that the ADNS in the dura mater is a kind of fascia, which implies that a fascia network may exist in the entire body and may have a specific function beyond the role of a supporting matrix (Lee and Soh, 2009; Lee, 2011; Jeon and Lee, 2013). In our judgment, the structure most related to the ADNS in terms of the fascia is the primo vascular system, which has been reported to exist in the brain (Lee et al., 2010; Dai et al., 2011; Lee and Lee, 2012). Because of the present data and the data in previous works, this newly-observed anatomical structure has emboldened us to view the entire fascia network as a new physiological organ with a specific function.

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Author contributions: *BCL and KBL designed the study and wrote the paper. HSL, DIK, SZY, YHR, IL and HGK conducted experiments. All authors approved the final version of this paper.*

Conflicts of interest: *None declared.*

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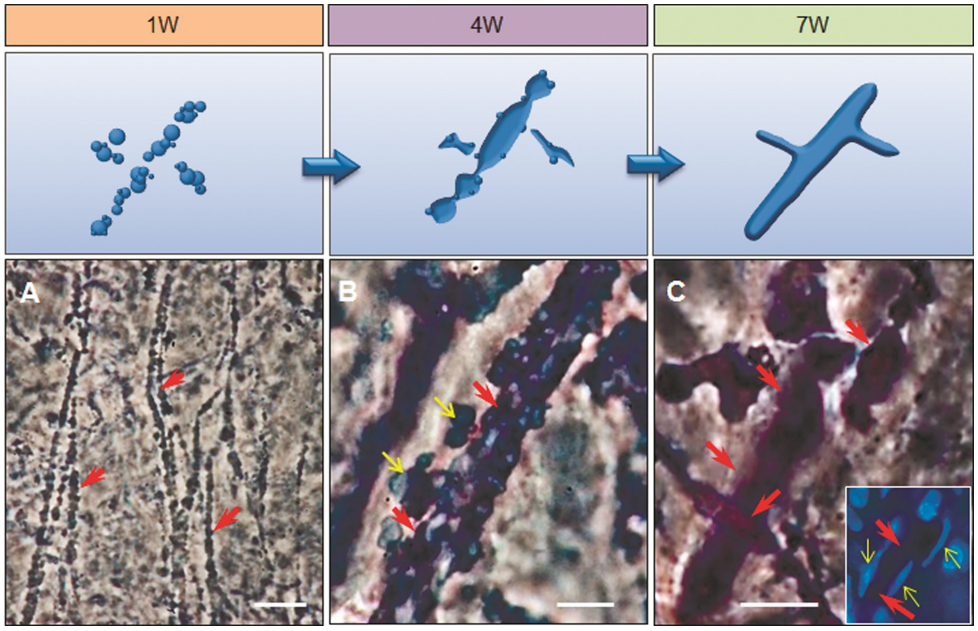

Figure 2 A hypothetical illustration and real images of age-dependent network structures (ADNSs) with age.
A hypothetical illustration of the morphological changes in the ADNSs with age is shown in the upper panel while phase contrast and fluorescent microscopy images corresponding to the illustration in the upper panel of the ADNSs in the dura mater of the rat brain are shown in the lower panel. The upper panel shows the following: 1 week (W): Scattered granules are arranged in a threadlike pattern. 4W: The granules are fusing together to become threadlike structures, with some granules still attached. 7W: All ADNSs have almost completely developed. The lower panel shows the following: (A) The ADNS has a bead-shaped form consisting of granules (red arrows). (B) Fused structures (red arrows) with attached granules (yellow arrows) exist. (C) The ADNS has become fully developed (red arrows) and contains rod-shaped nuclei (yellow arrows) that are stained by using 4′,6-diamidino-2-phenylindole, as shown in the inset. Scale bars: 20 μm in A, B, and 10 μm in C.

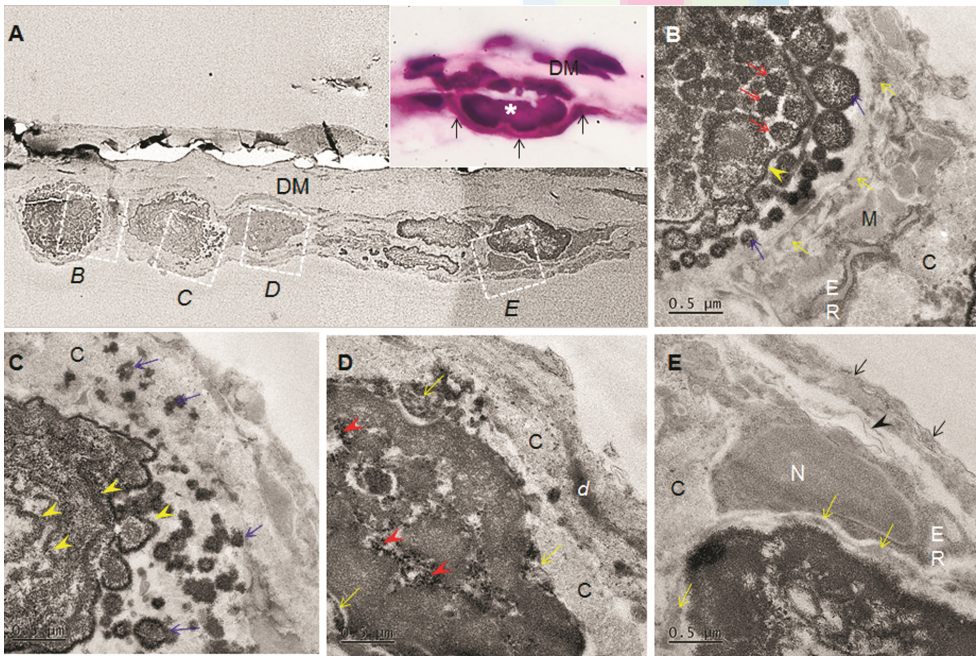

Figure 3 Light microscopy and transmission electron microscopy images of the age-dependent network structures (ADNSs) observed in the dura mater (DM) of the brains of 4-week-old rats.
(A) Low-magnification transmission electron microscopy image of the ADNS in the DM. The ADNS consisted of structures at different stages of development, with each dotted rectangle corresponding to a high-magnification image (B–E). The inset shows a hematoxylin-eosin-stained light microscopy image of a cross section of an ADNS (asterisk) above the border cell layer of the dura (arrows). (B) The ADNSs, which are enveloped by cellular cytoplasm (yellow arrow), contain high-electron-density granular clusters with (red arrows)/without (blue arrows) membranes (yellow arrowhead), where ER means endoplasmic reticulum, C means collagen fiber, and M means mitochondria. (C) The ADNSs consist of variously-sized granules (blue arrows) with multiple membranes (yellow arrowheads) scattered in collagen fibers. (D) The other ADNSs, which are enveloped by a dark cytoplasm (yellow arrows), contain tiny particles (red arrowheads) in irregular spaces, where d means a desmosome. (E) The ADNSs are enclosed by cytoplasm (yellow arrows) near the nucleus of a fibroblast (N). A cell junction (black arrowhead) exists between the fibrous dura and the border cell layer in the dura (black arrows).

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