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RESEARCH ARTICLE

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Novel Anatomic Structures in the Brain and Spinal Cord of Rabbit That May Belong to the Bonghan System of Potential Acupuncture Meridians

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Abstract
Novel threadlike structures of 20 to 40 μm in diameter were observed running afloat in the cerebrospinal fluid of the brain ventricles and the spinal central canal of a rabbit. We developed an effective *in situ* staining technique using hematoxylin to visualize the threadlike structure. The presence of the rod-shaped nuclei in the threadlike structure was confirmed by various nucleus specific staining dyes such as 4′,6′-diamidino-2-phenylindole, propidium iodide and yoyo-1. The threadlike structure was surrounded by a cellular membrane, whose presence was visualized by using 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate staining. The location, diameter, optical transparency and the presence of rod-shaped nuclei in and the surrounding membranes of the threadlike structure were consistent with a nerve Bonghan duct. The Bonghan duct was claimed to be the extension of the physical substance of acupuncture meridians and to be a distinct type of circulatory system present in mammals. Although Bonghan theory has not been reproduced for a long time, recently, some portions of the Bonghan duct network were confirmed in various organs in mammals including blood vessels, lymphatic vessels and enteric organs. The novel threadlike structure in the central nervous system, more specifically in brain ventricles, is one in a series of findings in an attempt to rediscover the Bonghan duct network.

1. Introduction

Acupuncture-meridian-like structures, also referred to as Bonghan ducts (BHDs), that form a novel circulatory system throughout an animal's body were first discovered by Bonghan Kim [1], but have been neglected for a long time. Only recently, several reports on observations of BHDs on the surface of the internal organs of rats and rabbits [2–4] revived the interest in the long forgotten work. The novel circulatory system is thought to be the anatomic basis of classical acupuncture meridians and consisted of several subsystems: superficial BHDs in the skin, intravascular BHDs inside large blood and lymphatic

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vessels, organ-surface BHDs on the surfaces of various internal organs and brain BHDs in brain ventricles.

The intravascular BHDs were confirmed by using an acridine-orange staining method [5,6] and those inside lymphatic vessels were observed by applying three different staining techniques [7–9]. Extensive histologic studies have been performed for organ-surface BHDs by using conventional staining methods [10], various electron microscopes [11] and immunohistochemistry [12].

In this article, we report on novel threadlike structures which are thought to be the brain BHDs in brain ventricles of rabbits. In neuroanatomy, brain ventricles are known to be sinuses containing cerebrospinal fluid (CSF) which has important roles in brain physiology [13–15]. There are also choroid plexuses [14,16,17] and Reissner fibers [18] in the ventricles. A choroid plexus is a cellular structure comprised of many blood capillaries. It generates cerebrospinal fluid and is embedded in the ependymal walls. Reissner fibers [19] are partially suspended in the cerebrospinal fluid and are made of material secreted from the subcommusura organ located in the circumventricular zone of the brain. A Reissner fiber is a fibrous structure inside the brain ventricle. It is not a cellular structure, but is comprised of accumulations of glycoprotein [18–20]. Because brain BHDs and Reissner fibers are both similar looking threadlike structures, we needed to clearly distinguish between them.

We detected brain BHDs in the third and the fourth ventricles that extended into and through the central canal of the spinal cord of a rabbit. The BHDs were afloat in CSF and did not adhere to the walls of the ventricles. After presenting the anatomic observations, we examined the novel threadlike structures to differentiate them from Reissner fibers and to identify them as BHDs by showing the presence of rod-shaped nuclei and outer membranes.

2. Materials and Methods

Ten New Zealand white rabbits (female, 12 weeks old) were obtained for this study from the Jung Ang Laboratory Animal Company of Korea. The animals were housed in a constant, temperature controlled environment (23ºC) with 60% relative humidity under a 12 hour light/dark cycle. All animals had *ad libitum* access to food and water. The procedures involving the animals and their care were in full compliance with institutional regulations and current international laws and policies (Guide for the Care and Use of Laboratory Animals, National Academy Press, 1996). The rabbits were anesthetized by using an intra-peritoneal injection with urethane (1.5 g/kg). Under deep anesthesia, the animals were decapitated without any perfusion. After a 1 hour freezing of the heads at −70ºC, the brains were isolated from the skulls as quickly as possible. In order to maintain the original shape of the brain, we put an ice pack beneath the isolated brain during dissection. The fourth ventricle was exposed and mildly cooled on an aluminum foil-covered ice pack, followed by careful dissection which was performed under a stereomicroscope. Hematoxylin filtered through a 0.2 μm pore-sized filter paper was poured into the exposed fourth ventricle of the brain drop by drop and kept there for 1 to 2 minutes. After hematoxylin *in situ* staining of the exposed fourth ventricle, 0.1 M phosphate buffered saline, pH 7.4, was applied drop by drop into the fourth ventricle. The pink colored choroid plexus in the fourth ventricle, which was readily identified by its many capillaries, was carefully removed. After staining and washing, a thin threadlike structure emerged along the midline of the fourth ventricle. The threadlike structure led to the third ventricle through the aqueduct. The *in situ* features were recorded using a CCD camera (Olympus DP70) coupled to a stereomicroscope (Olympus SZX12).

We cut the threadlike structure in the middle of the third ventricle and fixed it in neutral phosphate buffered formalin, pH 7.4, for study under a light-microscope. The threadlike structure specimen was stained by using a DNA specific dye, 4′,6′-diamidino-2-phenylindole (DAPI) and was observed by using phase-contrast microscopy. Confocal laser scanning microscopy (CLSM) was applied to optically examine sections of the threadlike structure. For a cross-sectioned image of the threadlike structure, we cut a 20 μm section of the specimen and stained the specimen with yoyo-1, a DNA-specific dye. After characterization of the nuclei of the threadlike structure, 10 μM of a phospholipid staining dye, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (DiI), was applied in order to visualize the outermost membrane surrounding the threadlike structure.

3. Results

In order to demonstrate the effectiveness of hematoxylin for making the novel threadlike structure visible, we carried out a stereoscopic observation with and without hematoxylin application. Figure 1 illustrates the pictures of the fourth ventricle of the same rabbit before and after hematoxylin staining. The picture before hematoxylin staining on the fourth ventricle, shown in Figure 1A, hardly shows any floating structure in the sulcus of the fourth ventricle. Hematoxylin staining and washing made

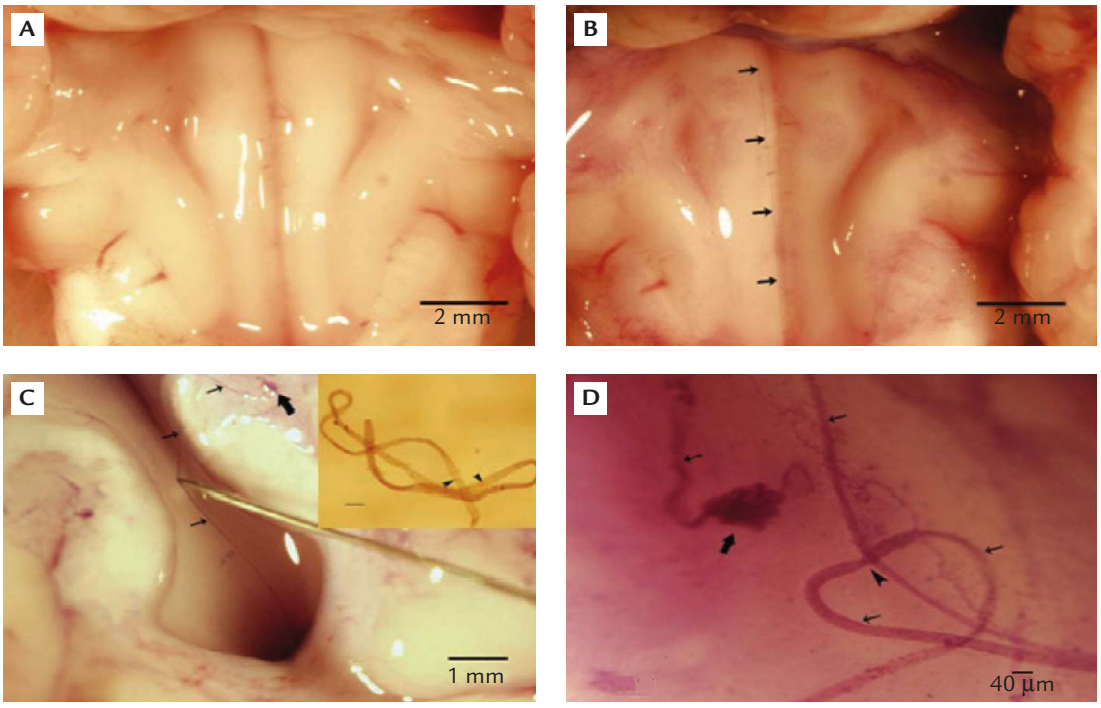

Figure 1 Identification of novel threadlike structures in the brain ventricles of rabbits. Stereomicroscopic images at the bottom of the fourth ventricle beneath the cerebellum of the same rabbit before (A) and after (B) hematoxylin application. No threadlike structure is visible in the panel A, but after hematoxylin administration and washing, the threadlike structure (arrows) emerged near the sulcus, as shown in panel B. (C) Stereomicroscopic image of a threadlike structure (arrow) in the aqueduct and the third ventricle of a rabbit brain after hematoxylin was applied and washed. It was lifted by using a needle to show that it was a floating tissue in the cerebrospinal fluid. The inset shows a wound state of the threadlike structure specimen, demonstrating its elastic nature. The overlapped regions show its optical transparency. There are two nodes (arrowheads). The scale bar is 60 μm. (D) Stereomicroscopic image of a threadlike structure (arrow) with a corpuscle (thick arrow) and a node (arrowhead). One end of the structure was cut at the front part of the third ventricle.

the novel threadlike structure (arrow) lying along the midline of the sulcus of the fourth ventricle visible as shown in Figure 1B.

Figure 1A illustrates the location of a threadlike structure against the ependymal walls of the third brain ventricle and of the cerebral aqueduct. We used an acupuncture needle to hold the threadlike structure (arrow) on the needle's tip. This demonstrated that the threadlike structure was afloat in the ventricle and in the aqueduct of the rabbit brain. According to previous studies, a BHD is known to have corpuscles and nodes along the duct. The inset in Figure 1C is a specimen that shows clearly the violet color of hematoxylin. The overlapped points of the wounded threadlike structure in the inset demonstrate its optical transparency. The threadlike structure was entwined after cutting due to its elastic nature. Figure 1D shows the features of a threadlike structure (arrow) stained with hematoxylin immediately after cutting at the front part of the third brain ventricle. Note the corpuscle structures (thick arrow) and a node (arrowhead).

As in the inset of Figure 1C, the first isolated threadlike structure, after cutting the threadlike structure in the third ventricle, is shown with two nodes (arrows). A stereomicroscopic image of a threadlike structure with a corpuscle and a node are visible in Figure 1D.

The presence of rod-shaped nuclei in the threadlike structure was shown by using various DNA-specific staining techniques. The phase contrast image of a threadlike structure exhibited three rod-shaped nuclei, which are indicated with circles in Figure 2A. They coincide with the three rod-shaped nuclei (arrow) stained by using DAPI and shown in Figure 2B. The lengths of the nuclei are about 15 μm. Figure 3A shows a confocal laser scanning microscopic (CLSM) image of a threadlike structure stained by using another DNA-specific dye, propidium iodide (PI). A combination of the differential interference contrast and the fluorescence images showed the location of the nucleus (arrow) in the threadlike structure. Figure 3B is an optically-sectioned CLSM image of the same threadlike structure. Figure 3 illustrates successive 10 μm depths from the lower surface of the threadlike structure. The signal got stronger as the section reached the middle part of the threadlike structure and became

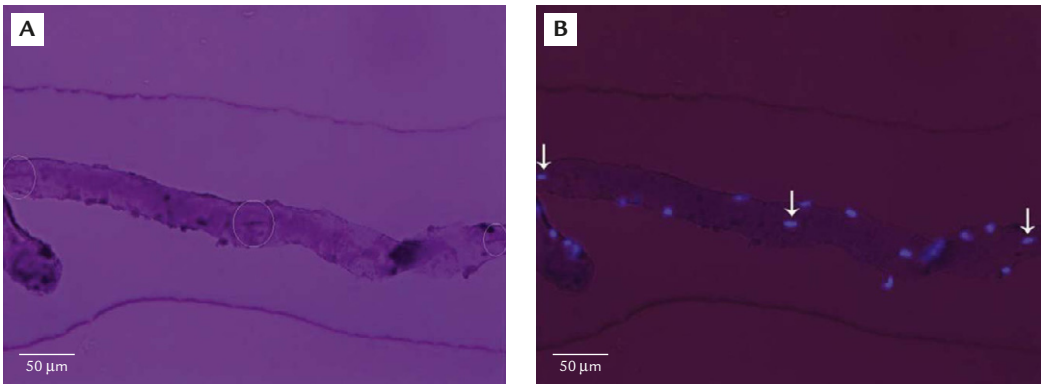

Figure 2 Detection of nuclei in a threadlike structure. Phase-contrast microscopic image of a threadlike structure specimen. The three circled bar-like objects coincide with the nuclei shown in (B). Fluorescence microscopic image of the same threadlike structure stained with 4′,6′-diamidino-2-phenylindole. Three nuclei (arrows) are shown.

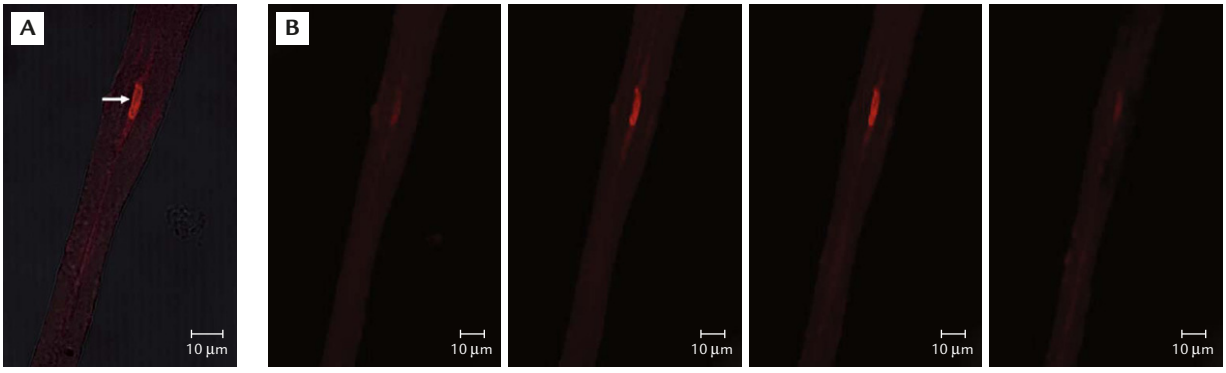

Figure 3 The visualization of nuclei of the threadlike structure. (A) Combined differential interference contrast and fluorescence microscopic image of a threadlike structure stained with propidium iodide. The nucleus is shown by an arrow. (B) Optically-sectioned images of the same threadlike structure at different depths. These four panels show successive 10 μm in depths from the lower surface of the threadlike structure. The signal increased as the section entered the threadlike structure and then became weaker at the upper surface, which implies that the nucleus was located inside the threadlike structure.

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Figure 4A is the CLSM of a threadlike structure (TS) stained by yoyo-1. The CLSM clearly demonstrated that there was a node indicated by an arrowhead as shown in the inset of Figure 1C. Figure 4B is a CLSM image of three cross-sectioned threadlike structures. This cross sectioned image showed that the yoyo-1-stained DNA signal was located in the center of one of three sectioned specimens.

The existence of cellular membranes in a threadlike structure was tested by using a DiI-staining method with CLSM. Figure 5 exhibits a bright DiI image at the outer boundary (arrows) of the threadlike structure.

Figure 6A shows a threadlike structure (arrows) in the central canal (arrowhead) of the spinal cord of a rabbit. The average diameters of the spinal cord, the central canal and the threadlike structure were 5000 μm, 150 μm and 30 μm, respectively. In order to demonstrate that the threadlike structures were not attached, but were freely floating in the CSF, we put a needle under the threadlike structure. The magnified view in Figure 6B shows that the threadlike structure was very transparent and that its thickness was uniform. Branches of the threadlike structure are indicated by arrowheads in Figure 6C. The presence of nuclei (arrows) in the threadlike structure is shown in Figure 6C and one of the nuclei is shown in a magnified view in Figure 6D. The length of the rod-shaped nucleus was about 15 μm, as expected for nuclei in a BHD.

4. Discussion

The first question regarding the novel threadlike structure is whether it is a genuine anatomic structure or an artifact. This skeptical question can be

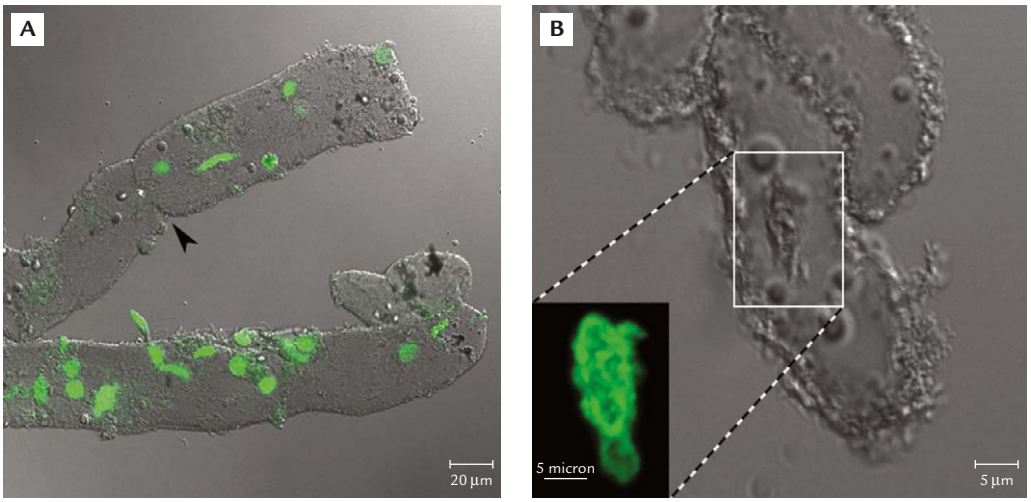

Figure 4 Confocal Laser Scanning Microscopic Image of Yoyo-1 stained threadlike structures. (A) Confocal Laser Scanning Microscopic Image of a threadlike structure (TS) stained by yoyo-1, a DNA-specific dye. Note that there is a clear node-like structure indicated by an arrowhead as shown in Figure 1C. (B) Confocal Laser Scanning Microscopic images of three cross-sectioned threadlike structures after cutting the folded specimen taken in the same site shown in Figure 1. Yoyo-1-stained DNA signal is shown in the center of one of three sectioned specimens.

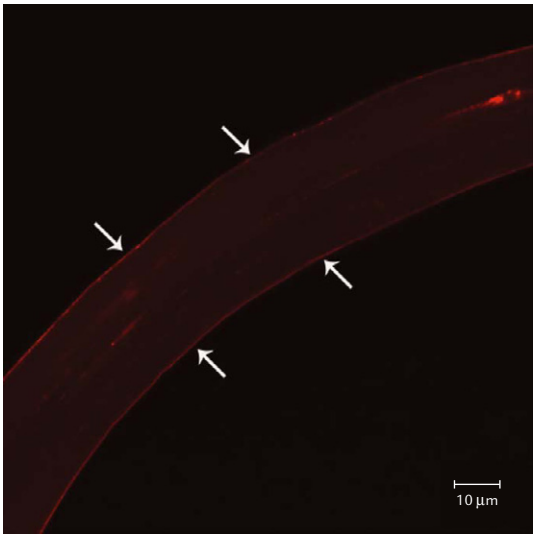

Figure 5 Threadlike structure surrounded by outer membrane. Confocal Laser Scanning Microscopic image of a threadlike structure stained with DiI, which stains phospholipids and reveals membrane structure. The outer surrounding membrane (arrow) of the threadlike structure is revealed.

answered based on its repeated observation and its structural properties. Presently, it has been detected in ten rabbits. There has been no problem in observing the structure with the techniques stated in the method section as long as the brains are intact. As Figures 2 and 3 demonstrate, the threadlike structure contains nuclei; therefore, it is a tissue formed of cells. Figure 4 demonstrates that the threadlike structure looks unique with node-like structures and DNA in the center of the structure. In addition, it is surrounded by a cellular membrane, as shown in Figure 5. This implies that it is an intact tissue, not tissue debris from the cerebral ventricle or aqueduct. Furthermore, the threadlike structure we observed was a long and uniform thread continuing from inside the third ventricle through the fourth ventricle to the central canal of the spinal cord, which is hard to explain away as an artifact.

Another question is why the threadlike structure has not been noticed before. One reason is its optical transparency. *In situ*, as shown in Figure 1A, the threadlike structure is extremely difficult to detect even when its presence is known. Only after hematoxylin staining is the threadlike structure visible. This simple staining method was found in our earlier experiments with another transparent threadlike structures on internal organs [21]. Another reason is the elasticity of the threadlike structure, as evidenced in the wound shape in the inset of Figure 1C. Unless the investigation is performed carefully, the threadlike structure may easily be cut or coil away, and thus be unobservable. A third reason may be attributed to the fact that threadlike structure is comprised of very rare cells. As Figure 2 shows the interval between the rod-shaped nuclei is about 300 μm. Therefore, the threadlike structure might easily be confused with tissue debris unless a careful examination is done on a specimen long enough to detect the nuclei.

A critical question is whether it is a Reissner fiber or novel threadlike structure [18,19]. Because a Reissner fiber is not a cellular object, but rather a fibrous material, it has neither nuclei nor an outer membrane. Thus our data, shown in Figures 3, 4 and 5, negates the possibility of the threadlike

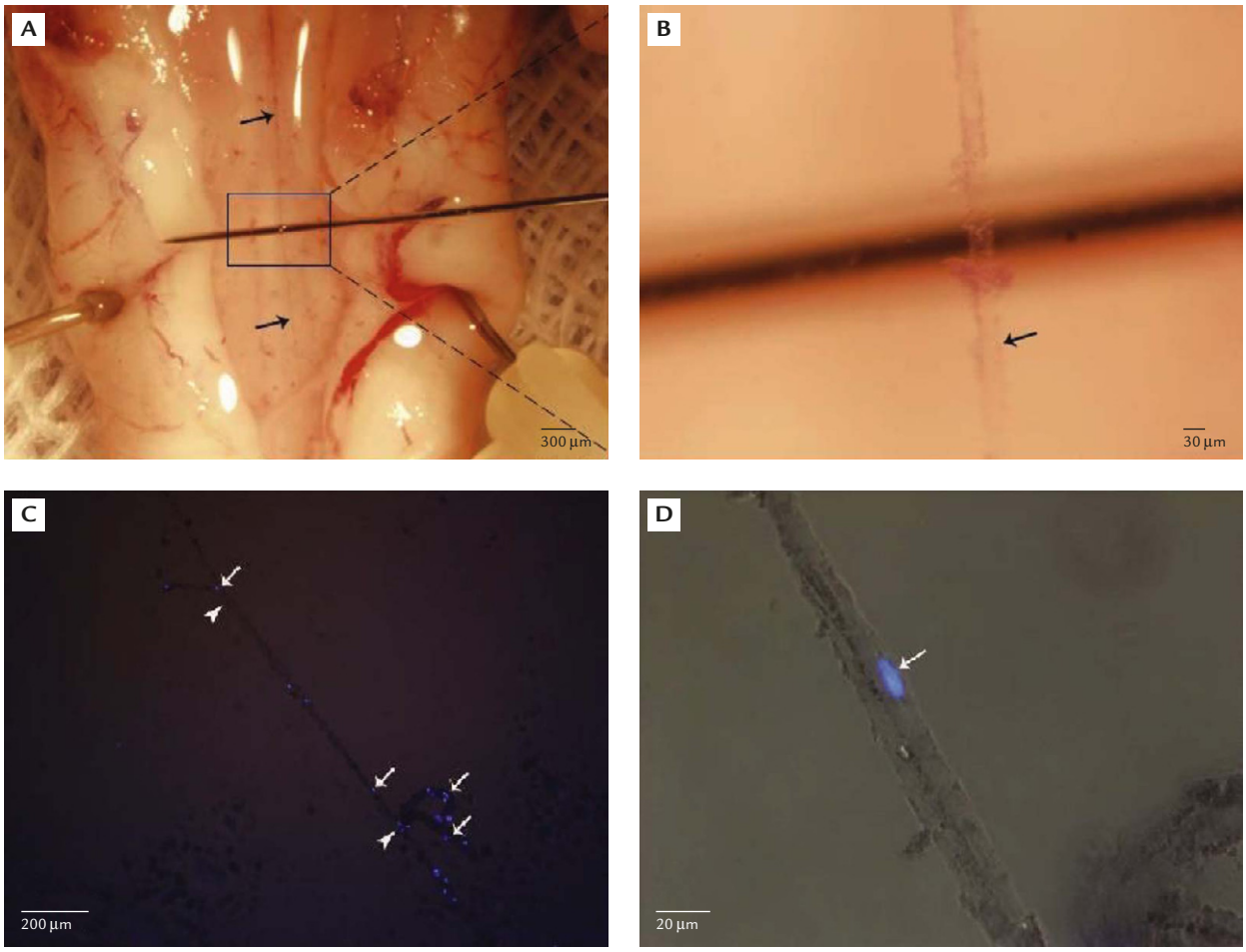

Figure 6 The threadlike structure in the central canal of the spinal cord. (A) Threadlike structure (arrow) inside an opened central canal of the spinal cord of a rabbit was lifted by using a needle. (B) The boxed area in the stereomicroscopic image in (A) was magnified to show the transparency of the threadlike structure (arrow). The black and gray horizontal bar is the needle. (C) The threadlike structure had branches (arrowheads) and rod-shaped nuclei (arrows) stained with 4′,6′-diamidino-2-phenylindole. These two features are consistent with those of Bonghan ducts. (D) A magnified view of the threadlike structure around a nucleus (arrow) shown in the panel (C).

structure being a Reissner fiber. There were nodes in the threadlike structure, which were absent in Reissner fibers. In addition, the thickness of a Reissner fiber is about 4 μm but that of a threadlike structure is about 30 μm. Furthermore, the corpuscles in Figure 1 and the branching of the threadlike structure in Figure 6 are consistent with a nerve BHD, to which we now turn our attention.

The fourth question is whether the threadlike structure is a BHD or not. The anatomic location and shape shown in Figure 1 agree with Bonghan Kim's description [22]. The characteristic hallmark of a BHD are the presence of rod-shaped nuclei of 10–20 μm in sizes as seen in the intravascular [6], lymphatic BHD [7–9] and organ-surface BHD [10]. Indeed, the features of threadlike structure shown in Figures 2 and 3 are consistent with these features. Another feature is the presence of an outer membrane that surrounds the BHD, as shown in Figure 5. With all this evidence, it is plausible that the threadlike structure is part of the BHD network, even though we need to reveal the connections to the full network in order to firmly establish the threadlike structure as a part of the BHD network.

It is important to know whether the threadlike structure is an isolated object in ventricles or it is connected to a threadlike structure outside the ventricles. In order to be a BHD, it must continue to the central canal of the spinal cord [22]. Indeed, as shown in Figure 6, we found that the threadlike structure extends through the central canal of the spinal cord. Interestingly Figure 6C showed that the nerve BHD in the central canal of a spinal cord had the branching points which were observed earlier by Bonghan Kim [22].

The research into the novel threadlike structures is only in its initial stages, so we can only offer some hypotheses on its physiologic functions. First of all, we consider the threadlike structure to be a part of the whole network of BHDs, which is a new

circulatory system of some liquid, including hyaluronan, adrenalin/noradrenalin hormones and other biochemicals [23]. This liquid has been claimed to play a critical role in the maintenance and the regeneration of tissues in various organs [22]. Because the BHD is thought to be a physical reality of acupuncture meridians, the threadlike structure may play a role in therapeutic treatment using acupuncture. These two aspects of the function of the threadlike structure suggest a mediator role between the brain and the acupuncture stimulation in the skin, which might be medically more specific than general communication by the nervous system. Elucidation of the detailed structure and function of the novel threadlike structure is worthwhile for further research.

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