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Visualizing the Calcitonin Gene-Related Peptide Immunoreactive Innervation of the Rat Cranial Dura Mater with Immunofluorescence and Neural Tracing

Jia Wang[*,1], **Dongsheng Xu**[*,1], **Jingjing Cui**[1], **Chen She**[1], **Hui Wang**[1], **Shuang Wu**[1], **Ling Zou**[1], **Jianliang Zhang**[1], **Wanzhu Bai**[1]

[1] Institute of Acupuncture and Moxibustion, China Academy of Chinese Medical Sciences

[*] These authors contributed equally

Corresponding Author

Wanzhu Bai

wanzhubaisy@hotmail.com

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Abstract

The aim of this study was to examine the distribution and origin of the calcitonin gene-related peptide (CGRP)-immunoreactive sensory nerve fibers of the cranial dura mater using immunofluorescence, three-dimensional (3D) reconstruction and retrograde tracing technique. Here, the nerve fibers and blood vessels were stained using immunofluorescence and histochemistry techniques with CGRP and fluorescent phalloidin, respectively. The spatial correlation of dural CGRP-immuoreactive nerve fibers and blood vessels were demonstrated by ~~three-dimensional~~ reconstruction. Meanwhile, the origin of the CGRP-immunoreactive nerve fibers were detected by neural tracing technique with fluorogold (FG) from the area around middle meningeal artery (MMA) in the cranial dura mater to the trigeminal ganglion (TG) and cervical (C) dorsal root ganglia (DRGs). In addition, the chemical characteristics of FG-labeled neurons in the TG and DRGs were also examined together with CGRP using double immunofluorescences. Taking advantage of the transparent whole-mount sample and 3D reconstruction, it was shown that CGRP-immunoreactive nerve fibers and phalloidin-labeled arterioles run together or separately forming a dural neurovascular network in a 3D view, while the FG-labeled neurons were found in the ophthalmic, maxillary, and mandibular branches of TG, as well as the C2-3 DRGs ipsilateral to the side of tracer application in which some of FG-labeled neurons presented with CGRP-immunoreactive expression. With these approaches, we demonstrated the distributional characteristics of CGRP-immunoreactive nerve fibers around the blood vessels in the cranial dura mater, as well as the origin of these nerve fibers from TG and DRGs. From the perspective of methodology, it may provide a valuable reference

for understanding the complicated neurovascular structure of the cranial dura mater under the physiological or pathological condition.

Introduction

The cranial dura mater is the outermost layer of meninges to protect the brain and contains plentiful blood vessels and different kinds of nerve fibers[1,2]. Many studies have shown that sensitized cranial dura mater may be the key factor leading to the occurrence of headaches, involving the abnormal vasodilation and innervation[3,4,5]. Thus, the knowledge of neurovascular structure in the cranial dura mater is important for understanding the pathogenesis of headaches, especially for migraine.

Although the dura innervation has been previously studied with the conventional immunohistochemistry, the spatial correlation of nerve fibers and blood vessels in the cranial dura mater were less studied[6,7,8,9]. In order to reveal the dural neurovascular structure in more detail, calcitonin gene-related peptide (CGRP) and phalloidin were selected as the markers for respectively staining the dural nerve fibers and blood vessels in the whole-mount cranial dura mater with immunofluorescence and fluorescent histochemistry[10]. It may be an optimal choice to obtain a 3D view of neurovascular structure. Additionally, fluorogold (FG) was applied on the area around middle meningeal artery (MMA) in the cranial dura mater to determine the origin of CGRP-immunoreactive nerve fibers, and traced to the trigeminal ganglion (TG) and cervical (C) dorsal root ganglia (DRGs), while the FG-labeled neurons were further examined together with CGRP using immunofluorescence.

The aim of this study was to provide an effective tool for investigating the neurovascular structure in the cranial dura mater for the CGRP-immunoreactive innervation and its origin. By taking the advantage of the transparent whole-mount dura mater and combining the immunofluorescence, retrograde tracing, confocal techniques, and 3D reconstruction, we expected to present a novel 3D view of the neurovascular structure in the cranial dura mater. These methodological approaches may be further served for exploring the pathogenesis of different headaches.

Protocol

This study was approved by the Ethics Committee of the Institute of Acupuncture and Moxibustion, China Academy of Chinese Medical Sciences (reference number D2018-09-29-1). All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, D.C., 1996). Twelve adult Sprague-Dawley male rats (weight 220 ± 20 g) were used in this study. Animals [license number SCXK (JING) 2017-0005] were provided by the National Institutes for Food and Drug Control.

1. Innervation of rat cranial dura mater

1. **Perfusions**
    1. Intraperitoneally inject an overdose of tribromoethanol solution (250 mg/kg) to the rat to induce euthanasia.

2. Once the breath stops, transcardially perfuse with 100 mL of 0.9% normal saline followed by 250-300 mL of 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4).

3. After perfusion, remove the head skin and open the skull to expose the dura mater and dorsal side of the brain. Then dissect out the cranial dura mater along the brainstem to the olfactory bulb in the whole-mount pattern (**Figure 1**). Perform post-fixation in 4% paraformaldehyde for 2 h, and then cryoprotect in 25% sucrose in 0.1 M PB for more than 24 h at 4 °C.

2. **Fluorescence immunohistochemistry for CGRP and phalloidin labeling**

NOTE: A combination of fluorescent staining of CGRP and phalloidin was applied to reveal the spatial correlation of dural nerve fibers and blood vessels in the rat cranial dura mater in the whole-mount pattern.

1. Rinse the cranial dura mater in 0.1 M PB for about 1 min.

2. Incubate the dura mater in a blocking solution containing 3% normal donkey serum and 0.5% Triton X-100 in 0.1 M PB for 30 min.

3. Transfer the dura mater into mouse anti-CGRP antibody (1:1000) in 0.1 M PB containing 1% normal donkey serum and 0.5% Triton X-100 overnight at 4 °C[11].

4. Wash the dura mater three times in 0.1 M PB the following day.

5. Incubate the dura mater in a mixed solution of donkey anti-mouse Alexa Fluor 488 secondary antibody (1:500) and phalloidin 568 (1:1000) in 0.1 M PB containing 1% normal donkey serum and 0.5% Triton X-100 for 1.5 h at room temperature (26 °C).

6. Wash the dura mater three times in 0.1 M PB.

7. Trim the edges and mount it on microscope slides (see **Table of Materials**).

8. Put on coverslips with 50% glycerin before observation.

3. **Observation and recording**

1. Observe the fluorescent samples under a fluorescent microscope or a confocal imaging system.

2. Take the images of the whole-mount dura mater by a fluorescent microscope equipped with a digital camera (4x, NA: 0.13), and use an exposure time of 500 ms. The image mosaics of the dura mater were completed with a software of fluorescent microscope (see **Table of Materials**).

3. Take images of the CGRP-immunoreactive nerve fibers and phalloidin-labeled blood vessels in the dura mater using a confocal microscope. The excitation and emission wavelengths were 488 nm (green) and 594 nm (red). The confocal pinhole is 110 (20x) and 105 µm (40x). The resolution of image capture is 640 x 640 pixels.

4. Capture 20 images in 2 µm frames of from each 40 µm thick section and perform single in-focus image integration with a confocal image processing associated software system for 3D analysis as follows: **Set Start Focal Plane | Set End focal plane | Set step size | Choose Depth Pattern | Image Capture | Z series.**

5. Use a photo editing software to adjust the brightness and contrast of images to optimize the visualization. Pay attention to not remove any data from the images.

2. Retrograde tracing study with FG

1. **Surgical procedures**

    1. Determine the coordinate area of interest in rat cranial dura mater.
    2. Prepare a 10 µL micro-syringe and test it with liquid paraffin.
    3. Anesthetize the rats with tribromoethanol solution (150 mg/kg) via intraperitoneal injection. Check for the depth in anesthesia by the lack of response to toe pinch.
    4. Shave the rat's head with an electric razor.
    5. Put blunt ear bars to the rat and place it on the stereotaxic device. Then put the mouth holder and apply ophthalmic ointment on the eyes.
    6. Clean the surgical site of the head skin using 75% ethanol.
    7. Make an incision along the midline of the scalp.
    8. Bluntly remove the periosteum and muscle tissues away from the skull using sterile cotton-tipped applicators (**Figure 1A**).
    9. Drill a small hole (~5-7 mm) using a burr drill with a round-tip bit (#106) on the left parietal and temporal bones above the ~~middle meningeal artery (MMA)~~[12], and make sure that the cranial dura mater was kept intact (**Figure 1B**).
    10. Build a bank around the hole with dental silicate cement to limit the spread of the tracer (**Figure 1C**).
    11. Add 2 µL of 2% FG into the hole around MMA with a 10 µL micro-syringe (**Figure 1D**).
    12. Cover the hole with a small piece of hemostatic sponge.
    13. Put a piece of paraffin film on the hole and seal the edges with bone wax to prevent the leakage of tracer and to avoid contaminating to the surrounding tissues.
    14. Suture the wound with sterile thread.
    15. Keep the rats in a warm area until they have fully recovered without any post-operative analgesics.
    16. Return the rats back to their cages.

2. **Perfusions and sections**

    1. After 7 survival days, perfuse these rats as mentioned above in the procedures in section 1.1.
    2. Dissect out the TG and C1-4 DRGs, then post-fix and cryoprotect them as above mentioned in section 1.1.3 (**Figure 1F**).
    3. Cut the TG and DRGs at the thickness of 30 µm on a cryostat microtome system in the sagittal direction and mounted on silane-coated glass slides.

3. **Double immunofluorescences for FG- and CGRP-labeling in the TG and DRGs**

    NOTE: Although the FG-labeling can be directly observed with UV illumination under mercury lamp without additional staining[13,14,15,16], the labeled neurons with FG were further examined in TG and cervical DRGs using double immunofluorescences with

FG and CGRP for revealing the origins of dural CGRP-immunoreactive nerve fibers in the TG and DRGs.

1. Circle the sections with the histochemical pen.
2. Incubate the sections for 30 min in a blocking solution containing 3% normal donkey serum and 0.5% Triton X-100 in 0.1 M PB.
3. Transfer the samples into the solution of rabbit anti-fluorogold (1:1000) and mouse anti-CGRP antibody (1:1000) in 0.1 M PB containing 1% normal donkey serum and 0.5% Triton X-100 overnight at 4 °C.
4. Wash the sections three times in 0.1 M PB the following day.
5. Incubate in a mixed solution of donkey anti-rabbit Alexa Fluor 594 (1:500) and donkey anti-mouse Alexa Fluor 488 (1:500) secondary antibody in 0.1 M PB containing 1% normal donkey serum and 0.5% Triton X-100 for 1.5 h at room temperature.
6. Wash and apply coverslips to the sections as the procedures in steps 1.2.6 and 1.2.8.

4. **Observation and recording**

1. Take images of the FG-labeled neurons in TG and DRGs under UV illumination by fluorescent microscope equipped with a digital camera.
2. Capture images of the FG- and CGRP-labeled neurons in TG and DRGs under a fluorescent microscope equipped with a digital camera.
3. Use the editing software to adjust the brightness and contrast of images and to add labels in the pictures.

Representative Results

Neurovascular structure of the cranial dura mater

After immunofluorescent and fluorescent histochemical staining with CGRP and phalloidin, CGRP-immunoreactive nerve fibers and phalloidin-labeled dural arterioles and connective tissues were clearly demonstrated throughout the whole-mount cranial dura mater in a 3D pattern (**Figure 2C,D,E,F**). It was shown that both thick and thin CGRP-immunoreactive nerve fibers run in parallel to the dural arterioles, around the vascular wall, or between the blood vessels (**Figure 2D,E,F**). By taking advantages of the 3D reconstruction, the morphology of dural arterioles and the spatial correlation of the dural CGRP-immunoreactive nerve fibers and arterioles could be clearly demonstrated from different perspectives.

Retrograde-labeled neurons in the TG and DRGs

Seven days after FG application on the region of MMA in the rat cranial dura mater (**Figure 3A**), the FG-labeled neurons were detected in TG and cervical DRGs on the ipsilateral side of the tracer application, which was directly observed under the UV illumination of the fluorescent microscopy (**Figure 3B,C**). FG-labeled neurons were found in all the three branches of TG with higher concentration on the ophthalmic (V1) and maxillary (V2) divisions, and with less on the mandibular division (V3) (**Figure 3B**). Meanwhile, some of the FG-labeled neurons were also observed in the C2-3 DRGs (**Figure 3C**).

In addition, double immunofluorescences were also performed with FG and CGRP on the sections of TG and cervical DRGs. According to the diameter of CGRP-immunoreactive neurons in TG and DRGs, most of them were primarily found in the small- and medium-diameter sensory neurons (<30 μm). Some of these type of CGRP-immunoreactive neurons were also labeled with FG in the TG and C2-3 DRGs, indicating that CGRP-immunoreactive nerve fibers in the cranial dura mater originated from these subpopulation of sensory neurons in the TG and DRGs (**Figure 4**).

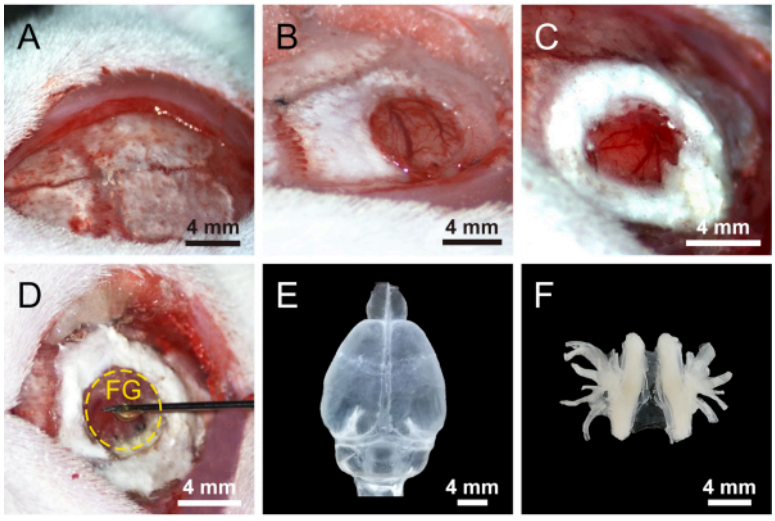

Figure 1: Photographs of main experimental views in the present study. (**A**) An incision along the midline of the scalp. (**B**) A hole above the cranial dura mater showing the middle meningeal artery (MMA). (**C**) A small bank around the hole circled with dental silicate cement for the application of tracer onto the cranial dura mater. (**D**) Application of fluorogold (FG) into the hole with micro-syringe. (**E**) The transparent whole-mount dura mater. (**F**) The outside view of the trigeminal ganglion (TG). Please click here to view a larger version of this figure.

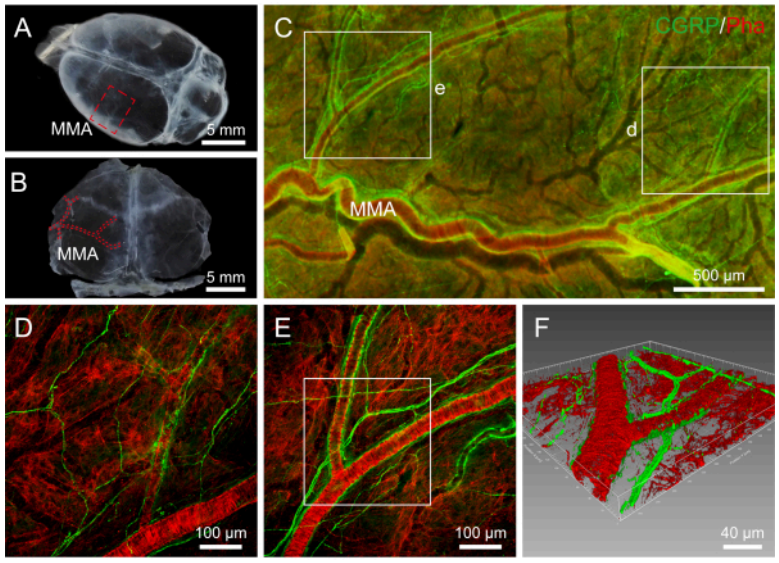

Figure 2: Correlation between calcitonin gene-related peptide (CGRP)-immunoreactive nerve fibers and phalloidin (Pha)-labeled arterioles on the whole-mount cranial dura mater. (**A**) The transparent whole-mount dura mater. (**B**) The whole-mount dura mater was flattened and mounted on the slide. (**C**) Distribution of CGRP-immunoreactive nerve fibers and Pha-labeled blood vessels along the middle meningeal artery (MMA). (**D,E**) The magnified photos from the same areas of D and E in panel C. (**F**) The magnified and adjusted images from panel E with the frame in a 3D pattern. Please click here to view a larger version of this figure.

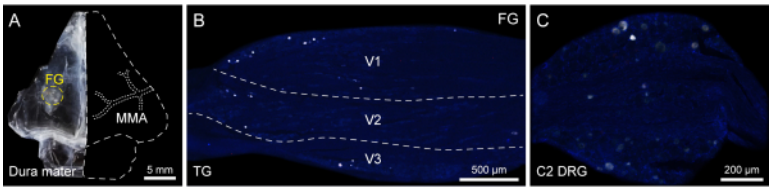

Figure 3: Distribution of fluorogold (FG)-labeled neurons in the trigeminal ganglion (TG) and cervical (C) dorsal root ganglion (DRG) under UV illumination. (**A**) The region of dura mater with FG application. (**B**) Distribution of FG-labeled neurons in the ophthalmic (V1), maxillary (V2), and mandibular (V3) branches of the TG. (**C**) Distribution of FG-labeled neurons distributed in the C2 DRG. Please click here to view a larger version of this figure.

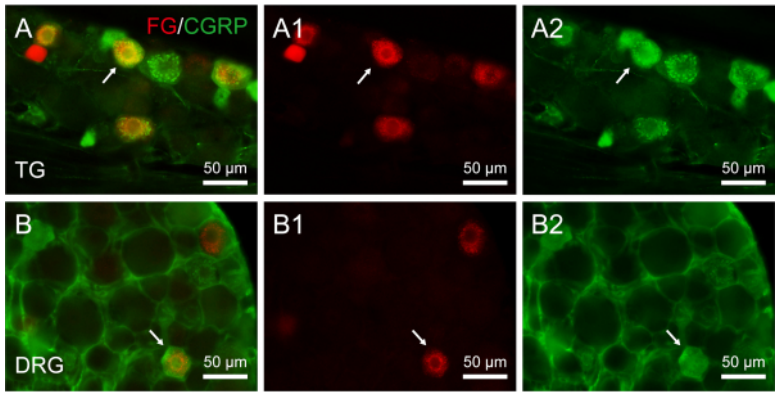

Figure 4: The representative photographs showing the labeled sensory neurons in trigeminal ganglion (TG) and cervical dorsal root ganglion (DRG) by using double immunofluorescences with fluorogold (FG) and calcitonin gene-related peptide (CGRP). (**A,B**) The labeled neurons with FG, CGRP, and both FG and CGRP were demonstrated in red, green, and yellow, respectively in TG (A) and DRG (B). ~~A1-B1~~, (A2-B2): Panels A and B were separately showed with FG-labeling (A1, B1) and CGRP-labeling (A2, B2). Please click here to view a larger version of this figure.

Discussion

In this study, we have successfully demonstrated the distribution and the origin of CGRP-immunoreactive nerve fibers in the cranial dura mater using immunofluorescence, 3D reconstruction and neural tracing approaches with CGRP antibody and FG neural tracer, providing the histological and chemical evidences to better understand the dural neurovascular network.

As it was known, CGRP plays a critical role in the pathogenesis of migraine[4,17]. It was shown that increased CGRP can lead to vasodilatation and neurogenic inflammation to cause the peripheral and central sensitization along the trigeminal pathway[4,18]. CGRP-immunoreactive nerve fibers belong to the unmyelinated peptidergic sensory axons responsible for the transportation of nociceptive signals[19]. Being consistent with previous studies, here, we clearly demonstrated the distribution of the CGRP-immunoreactive nerve fibers in the cranial dura mater and traced their origin from small- and medium-diameter sensory neurons in the TG and DRGs. These cellular structures could be the sources for synthesizing and releasing CGRP. On the other hand, phalloidin is a specific probe for filamentous actin (F-actin) that is abundant in the smooth muscular and endothelial cells. As a proper candidate, phalloidin was used for labeling the vascular structures and connective tissues[10,20,21]. Our recent study has shown that, in contrast to alpha smooth muscle actin and CD31, phalloidin is more reliable and sensitive for staining dural arterioles, and is the optimal to combine with CGRP for demonstrating the cranial neurovascular network in detail, which has been published eslewhere[10,21].

Neural tract tracing technique is an important tool to investigate the neural origin and termination. In the present study, FG was used for retrograde tracing the origin of CGRP-immunoreactive nerve fibers in the cranial dura mater. Since

the cranial dura mater is a thin membrane, the tracer cannot be applied conveniently by the way of injection. Instead, FG was directly added onto the region around MMA in the cranial dura mater according to the method that had been introduced previously[22,23], but care must be taken to keep the dura mater intact. Besides, the effort was made to prevent the leakage of FG to the adjacent tissues. Because the FG-labeling can be directly observed under UV illumination of mercury lamp[24], by this approach, we checked the site of FG application; it was found that besides the neural propagation, FG was limited in the region circled with dental silicate cement without contaminating the surrounding tissues. The other advantage is that FG-labeled neurons can also be stained in the expected color using immunofluorescence with FG antibody, making it more convenient to be used together with other biomarkers[14,15,16]. Through this study, we proved that FG not only fits for retrograde tracing the dural innervation but is also a proper candidate to combine with CGRP for determining the chemical characteristics of FG-labeled neurons.

It should be noted here that present methods are preferably used for young animals. At the early stage of rat, the cranial dura mater is more transparent in the whole-mount style. This feature makes it more convenient to visualize the neurovascular structure of the cranial dura mater in a 3D pattern without further transparent treating.

In summary, the present study provides a valuable approach to effectively explore the innervation of the cranial dura mater from the sensory neurons in the TG and cervical DRGs, especially the subtype of small- and medium-diameter sensory neurons with CGRP-immunoreactive expression. From the perspective of methodology, it may provide a valuable reference for further investigating the other kinds of nerve fibers in the cranial dura mater, as well as their origins.

Disclosures

The authors have nothing to disclose.

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