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·Original Article·

Evaluation of three tracers for labeling distal cerebrospinal fluid-contacting neurons

Fang Zhou[1,2,3,4,*], Jiayou Wang[2,*], Hongxing Zhang[1,2,*], He Liu[1,2], Guangping Zhao[2], Cuihua Zu[2], Xiaoxing Lu[2], Licai Zhang[1,2]

[1]*Department of Anesthesiology, The First Clinical College, China Medical University, Shenyang 110001, China*

[2]*Jiangsu Province Key Laboratory of Anesthesiology,* [3]*Jiangsu Key Laboratory of Brain Disease Bioinformation,* [4]*Nursing School, Xuzhou Medical College, Xuzhou 221002, China*

[*]These authors contributed equally to this work.

Corresponding author: Licai Zhang. E-mail: licaizhang001@163.com

ABSTRACT

It has been reported that distal cerebrospinal fluid-contacting neurons (dCSF-CNs) can be detected by immunohistochemical assay using cholera toxin subunit B-conjugated horseradish peroxidase (CB-HRP). In the present study, another two methods with CB alone or CB-conjugated FITC (CB-FITC) were used, and the results from the three methods were compared. Adult Sprague-Dawley rats were randomly divided into three groups with CB-HRP, CB or CB-FITC. Tracers were diluted to 30% in artificial cerebrospinal fluid and injected separately (in a volume of 3 μL) into the lateral ventricle. Animals from the CB-HRP and CB groups were perfused 48 h after surgery while animals from the CB-FITC group were perfused 1, 3, 6, 12, 24 or 48 h after surgery. The brain was sectioned (40 μm) for immunofluorescence and five sections with positive neurons were selected from each rat for neuron counting. Three clusters of positive neurons in a 'Y-like' distribution were detected ventral to the cerebral aqueduct of rats from the three groups. No significant difference was observed among the quantitative data. In the CB-FITC group, stable staining was detected even at 6 h after injection. Taken together, lateral ventricle injection of CB/CB-FITC is a useful method for labeling dCSF-CNs in rats. The CB-FITC method makes dCSF-CNs labeling much simpler and more convenient.

Keywords: distal cerebrospinal fluid-contacting neurons; cholera toxin subunit B-conjugated horseradish peroxidase; cholera toxin subunit B; cholera toxin subunit B-conjugated FITC; label

INTRODUCTION

Cerebrospinal fluid-contacting neurons (CSF-CNs), which contact the CSF and the brain parenchyma[1], are of various cytological types and are located in different regions. CSF-CNs are divided into three categories, intra-ependymal neurons that line the walls of the ventricles and the central canal of the spinal cord, supra-ependymal neurons that are closely adjacent to the ependyma, and distal cerebrospinal fluid-contacting neurons (dCSF-CNs)[2,3]. The dCSF-CNs consist of three clusters of neurons in a 'Y-like' distribution, whose somata are located ventral to the cerebral aqueduct while some of their dendritic processes extend into the CSF. This is the 'cerebrospinal fluid-contacting nucleus'[4]. The unusual location of these neurons suggests that their activity may be associated with the CSF, such as non-synaptic signal transmission by releasing or absorbing an unknown bioactivator[5]. Due to the special structure and function of CSF-CNs, for the past 20 years, research has been focused on the molecular mechanisms within CSF-CNs underlying pathological processes such as morphine dependence and tolerance, nociception and chronic stress[4,6-8].

However, without a specific tracing method, it is difficult to label the dCSF-CNs in the brain parenchyma,

let alone study their biological functions. Researchers have been pursuing a reliable method for detecting these unique neurons for many years and in 1992, our laboratory succeeded in introducing a reliable labeling method by injecting cholera toxin subunit B-conjugated horseradish peroxidase (CB-HRP) into the lateral ventricle (LV). This method distinguishes dCSF-CNs from other neurons and labels them clearly in rat brain using histochemistry[2]. The dCSF-CNs are black under light microscopy when treated with tetramethylbenzidine-sodium tungstate (TMB-ST). In recent years, classic labeling methods have been developed for immunofluorescence[3,6]. Previous studies suggested that HRP and other markers such as radiolabeled substances cannot distinguish the dCSF-CNs specifically. As a result, we hypothesized that LV injection of CB alone might be another successful method for labeling dCSF-CNs.

In order to make it more convenient, we used CB-FITC (CB conjugated with the green-fluorescent dye FITC) as a direct tracer. However, the CB-FITC complex is different in structure from CB and CB-HRP. This study aimed to test whether CB and CB-FITC identify dCSF-CNs as reliably as CB-HRP.

MATERIALS AND METHODS

Animals

Sprague-Dawley rats (male, SPF grade, 250 ± 50 g) were from the laboratory animal center of Xuzhou Medical College. They were maintained in a light- and temperature-controlled room (12-h dark/light cycle with light on from 08:00–20:00, 23 ± 1°C) for at least two days prior to experiments. All experiments were approved by the Animal Care and Use Committee of Xuzhou Medical College and complied with the ethical guidelines of the International Association for the Study of Pain.

CB-HRP, CB or CB-FITC Administration

Animals were randomly divided into three groups: CB-HRP, CB and CB-FITC (n = 6/group). Rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and immobilized in a stereotaxic frame (Digital Stereotaxic Apparatus; Harvard Bioscience Co., Holliston, MA). Then, 3 μL of 30% CB-HRP (Sigma, St. Louis, MO), 30% CB (Sigma) or 30% CB-FITC (List Biological Laboratories Inc., Campbell, CA) (all diluted in artificial cerebrospinal fluid) was injected into the right LV according to stereotaxic coordinates[2] (bregma: −1.2 ± 0.4 mm, depth: 3.2 ± 0.4 mm, right of median sagittal plane: 1.4 ± 0.2 mm).

Immunofluorescent Staining and Image Analysis

At 1–48 h after tracer injection, rats were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and intracardially perfused with phosphate-buffered saline (0.01 mol/L PBS, pH 7.4), followed by 4% paraformaldehyde in phosphate buffer (0.1 mol/L, pH 7.4). The brain was removed immediately and postfixed in 4% paraformaldehyde at 4°C overnight, then cryoprotected in 30% sucrose at 4°C for 24–48 h. Coronal brain sections (40 μm) were cut on a cryostat (Leica CM1900, Germany) at −20°C.

Sections from the CB-HRP/CB group were collected in PBS. After three washes in 0.01 mol/L PBS, sections were incubated in PBS containing 0.3% Triton X-100 (PBST) for 30 min at 37°C, and for 48 h in goat anti-cholera toxin B-subunit antiserum (1:200, Calbiochem, San Diego, CA) at 4°C. Sections were rinsed in PBS and incubated in donkey anti-goat IgG-rhodamine (1:200, Santa Cruz, Dallas, TX) for 2 h in the dark at room temperature, after which the sections were rinsed, mounted, coverslipped and stored in the dark at −20°C. The dCSF-CNs labeled with rhodamine were identified by laser scanning confocal microscopy (TCS SP2, Leica, Germany) and sections from the CB-FITC group were examined after they were rinsed, mounted and coverslipped, without the immunoreaction procedure.

Five sections with labeled neurons were selected from each rat and the numbers of CB-HRP, CB and CB-FITC-positive neurons were counted using a digital image analyzer (Image-Pro plus Version 6.0, Media Cybernetics, Bethesda, MA) regardless of the staining intensity.

Statistical Analysis

Data are expressed as mean ± SD. Statistical com-parisons of more than two groups were performed using one-way ANOVA followed by a *post hoc* Tukey's test. $P < 0.05$ was considered statistically significant.

RESULTS

Image Analysis and Morphology

We previously reported that intra-LV injection of CB-HRP

can be used to identify dCSF-CNs in rats[2]. In conjunction with the earlier work, the present study showed that in all the rats treated with CB-HRP, CB alone or CB-FITC, the three clusters of dCSF-CNs were found in a 'Y-like' ditribution in the ventral aqueductal region, and most of the neurons were multipolar (Fig. 1).

Neuron Counts

The positive cells in the three groups were analyzed as

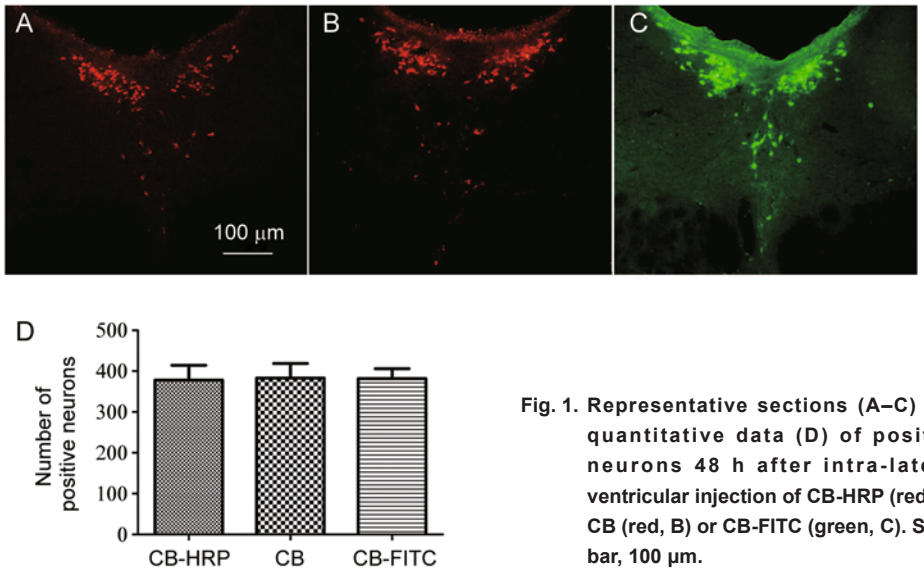

Fig. 1. Representative sections (A–C) and quantitative data (D) of positive neurons 48 h after intra-lateral ventricular injection of CB-HRP (red, A), CB (red, B) or CB-FITC (green, C). Scale bar, 100 μm.

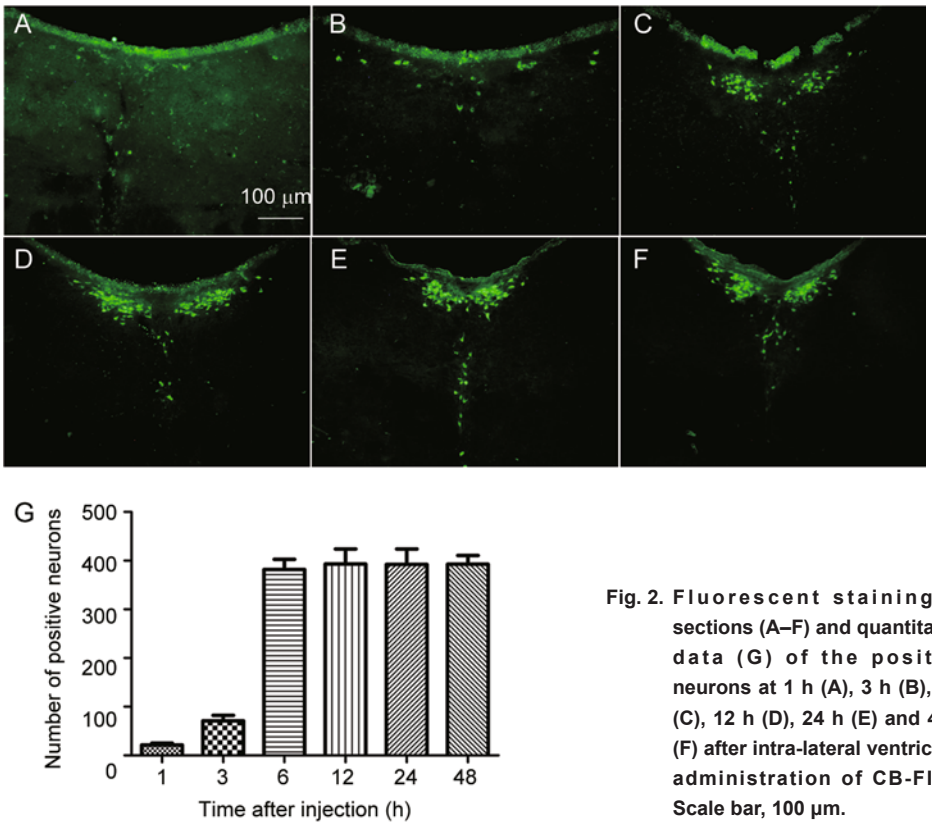

Fig. 2. Fluorescent staining of sections (A–F) and quantitative data (G) of the positive neurons at 1 h (A), 3 h (B), 6 h (C), 12 h (D), 24 h (E) and 48 h (F) after intra-lateral ventricular administration of CB-FITC. Scale bar, 100 μm.

previously described[6]. The labeled neurons in each group of six rats were counted on five positive neuron-containing sections from each rat. The quantitative data were 384 ± 38 in the CB-HRP group, 390 ± 37 in the CB group, and 388 ± 25 in the CB-FITC group (Fig. 1D). There was no significant difference among the three groups.

However, the CB-FITC labeling showed differential temporal dynamics. Few neurons were marked at 1 h and 3 h after administration, while fluorescent staining was stable at 6, 12, 24 and 48 h. There was no significant difference among the latter four time points (Fig. 2).

DISCUSSION

In light of the unique structure and location of dCSF-CNs, they may play an important role in the CSF such as signaling transmission, substance transport, and functional modulation. However, little is known due to methodological limitations such as specific molecular labeling methods. Therefore, to elucidate their function, it is essential to identify and distinguish the dCSF-CNs from other neurons in the brain.

In early studies, researchers used HRP or autoradiography to mark dCSF-CNs[9-12]. However, these methods were subsequently discredited since both penetrate freely through the ventricular ependyma into the brain parenchyma, resulting in non-specific staining and decreased affinity in labeling for dCSF-CNs. Studies of dCSF-CNs had been limited until 1992, when Zhang *et al.* found that CB-HRP, which cannot pass through the spaces between the ependyma into the parenchyma, was still able to specifically label dCSF-CNs[2]. In 2008, Lu *et al.* confirmed the method by comparing the staining with the TMB-ST method, immunofluorescent staining, and immuno-electron microscopic procedures after injecting CB-HRP into the LV in rats[3].

Based on the previous study, we believe that the key factor that identifies the dCSF-CNs is CB rather than HRP. However, CB is widely known as a peripheral tracer. It is a non-toxic receptor-binding subunit of cholera toxin which, in the periphery, combines with ganglioside GM1 receptors in the cell membrane[13], which then enter the cell body by receptor-mediated endocytosis (the receptor-ligand complex can be retrogradely transported from the axon terminals to the cell body and dendrites or anterogradely from the cell body to the axon terminals)[14]. We next asked if the same kind of staining occurred when CB alone was injected into the LV. In this study, immunofluorescent staining was used to compare the efficiency of the tracers CB-HRP and CB. The results suggested that LV injection of CB alone reliably and specifically labels dCSF-CNs. Furthermore, to make it simpler and more convenient, CB-FITC was administered into the LV as a direct fluorescent marker. Our results suggested that CB-FITC is as efficient in marking dCSF-CNs as CB and CB-HRP, and no significant difference was found in the quantitative data.

Considering the time taken for CSF circulation and retrograde transport, it would take hours for CB-FITC to be transported into dCSF-CNs. Therefore, few neurons were labeled at 1 h and 3 h after CB-FITC administration into the LV, while dCSF-CNs were reliably identified at 6, 12, 24 and 48 h later. Since no significant difference was observed among these four times, we believe that 6 h of survival might be applicable for the other staining methods.

In summary, the results of this study showed that LV injection of CB/CB-FITC is a reliable method for labeling dCSF-CNs in rats, and CB-FITC is simpler and more convenient than previous methods since the direct fluorescent staining simplifies the experimental process and reduces the experiment duration. In short, the present study provides a novel and convenient way to distinguish dCSF-CNs, and serves as an important methodological basis for studying their structure and function.

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