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

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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, 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). 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’. 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. 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.

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 (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 PBST and incubated in donkey anti-goat IgG-rhodamine (1:200, Santa Cruz, Dallas, TX) for 4 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 comparisons 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