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Original Article

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Neurochemical Properties of Dental Primary Afferent Neurons

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The long belief that dental primary afferent (DPA) neurons are entirely composed of nociceptive neurons has been challenged by several anatomical and functional investigations. In order to characterize non-nociceptivepopulation among DPA neurons, retrograde transport fluorescent dye was placed in upper molars of rats and immunohistochemical detection of peripherin and neurofilament 200 in the labeled trigeminal ganglia was performed. As the results, majority ofDPA neurons were peripherin-expressing small-sized neurons, showing characteristic ofnociceptive C-fibers. However, 25.7% of DPA were stained with antibody against neurofilament 200, indicating significant portion of DPA neurons are related to large myelinated Aβ fibers. There were a small number of neurons thatexpressed both peripherin and neurofilament 200, suggestive of Aδ fibers. The possible transition of neurochemical properties by neuronal injury induced by retrograde labeling technique was ruled out by detection of minimal expression of neuronal injury marker, ATF-3. These results suggest that in addition to the large population of C-fiber-related nociceptive neurons, a subset of DPA neurons is myelinated large neurons,which is related to low-threshold mechanosensitive Aβ fibers. We suggest that these Aβ fiber-related neurons might play a role as mechanotransducers of fluid movement within dentinal tubules.

Key words: nociceptor, trigeminal ganglion, tooth pain, peripherin, NF200, ATF3

INTRODUCTION

Dental afferent neurons are unique in that they are exclusively composed of nociceptive sensory neurons [1], which has gathered tremendous interest for physiological, anatomical and psychological investigation of structure and function of nociceptive sensory system [2, 3]. The assumption that tooth pulp neurons comprise only nociceptors is based on the observation that human tooth pulp produces only pain in response to noxious and non-noxious physical stimuli [4-6]. However, several reports suggest that tooth pulp neurons are not entirely consisted of nociceptors, based on observation of non-nociceptive sensory perception in response to subthreshold electrical stimulation of human teeth [2, 7, 8]. More recently published reports argue that majority of dental afferent neurons are non-nociceptive mechanoreceptors [9-12]. Therefore, it is crucial to determine

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whether dental primary afferent neurons are purely nociceptors or not in order to understand the sensory system of oral cavity.

In an attempt to characterize the nociceptive and non-nociceptive neurons among the dental primary afferent neurons, we have previously investigated the expression of several nociceptor-related receptors by electrophysiological analysis, single cell RT-PCR and immunohistochemical studiesand found out that not all of the dental primary afferent neurons expressed nociception-related receptors [13, 14]. To confirm the non-nociceptive population of dental primary afferent neuronsfurther, we investigated the neurochemical properties of retrogradely labeled dental primary afferent neurons in this study. Nociceptive neurons have smaller diameter unmyelinated or thinly myelinated axons and exhibit slower conduction velocity, whereas non-nociceptive large-diameter thickly myelinatedneurons have higher conduction velocity. Since it was difficult to measure the conduction velocity of single fibers, slowly conducting neurons and fast conducting neurons wasdistinguished by detecting differential neurochemical properties. In this study, we investigated the expression of peripherin and neurofilament 200 as markers for small neurons and myelinated neurons, respectively [15-17], in retrogradely labeled trigeminal ganglion neurons to determine whether dental primary afferent neurons are pure nociceptors or not. In addition, we examined whether our retrograde labeling methods caused neuronal damage, which could have affected the neurochemical properties of dental primary afferent neurons.

MATERIALS AND METHODS

Animals

All surgical and experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at Seoul National University. Animal treatments were performed according to the Guidelines of the International Association for the Study of Pain. Male Sprague-Dawley rats (approximately weighing 180~200 g at the time of surgery) were used. Rats were housed at a temperature of 23±2°C with a 12-hour light-dark cycle and fed food and water ad libitum. The animals were allowed to habituate to the housing facilities for 1 week before the experiments, and efforts were made to limit distress to the animals.

Retrograde labeling

Trigeminal ganglion neurons were retrograde-labeled with a fluorescent dye, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbo cyanine perchlorate (DiI; Molecular Probes, Eugene, OR, USA), as previously described [13, 18-20]. Briefly, cavities were drilled in 2 or 3 left and right upper molars and DiI crystals were placed into the cavity. Pulp exposure was avoided or minimized during the cavity preparation. Temporary sealing material (Caviton®; GC Corporation, Tokyo, Japan) was placed on the DiI to prevent spread of leaked tracer.Rats were fixed for immunohistochemical analysis two weeks after application of DiI.

Immunofluorescent staining

Rats were perfused with physiological saline and sequentially with 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4) at14 days after DiI placement. The trigeminal ganglion was removed and immersed in the postfixative at 4°C overnight and then transferred to 10% to 30% sucrose in PBS for 48 hours. Serial frozen transverse sections (14 μm thickness) were mounted on gelatin-coated slides. All immunohistochemical procedures were performed at room temperature unless otherwise stated. Slides were washed in PBS and then incubated in the blocking solution containing 5% normal goat serum, 2% BSA, 2% FBS, and 0.1% Triton X-100 for 1 hour at room temperature. The sections were incubated overnight at 4°C with either mouse anti-neurofilament 200 antibody (1:1,000; Sigma-Aldrich, St.Louis, MO, USA), goat anti-peripherin antibody (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or rabbit anti-ATF3 antibody (1:500; Santa Cruz Biotechnology, Inc.). Sections were then washed and incubated for 1 hour at room temperature with a FITC-conjugated donkey anti-mouse IgG(H+L) antibody (1:200; Jackson ImmunoResearch, West Grove, PA, USA), FITC conjugated donkey anti-goat IgG(H+L) antibody (1:200; Jackson ImmunoResearch) and FITC conjugated donkey anti-rabbit IgG(H+L) antibody (1:200; Jackson ImmunoResearch) for 1 hour. The sections were mounted with Vectashield® (Vector Laboratories, Inc., Burlingame, CA, USA) and visualized using a confocal microscope using the appropriate filter sets (FV-300; Olympus, Tokyo, Japan). 18 to 24 sections were used for counting the number of neurons that were stained with each antibody.

Inferior alveolar nerve transection injury

Transection injury of the inferior alveolar nervewas performed in accordance to the original description of Kim et al. [21]. Briefly, after anesthetizing the animals with sodium pentobarbital (30 mg/kg, ip), the facial skin over the left masseteric muscle was cut and the mandibular bone was exposed. The inferior alveolar nervewas exposed by carefully removing the surface bone covering the inferior alveolar nerve. The left inferior alveolar nervewas transectioned where the nerve trunk lies just beneath the coronoid process, and cutaneous tissues were then sutured. The contralateral sides were left intact in all rats.

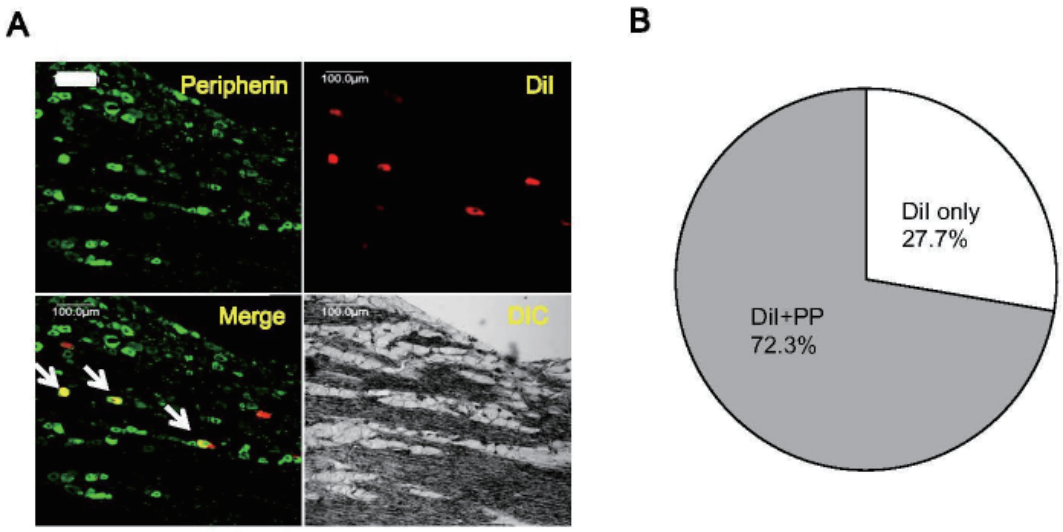

Fig. 1. Expression of peripherin in DiI labeled dental primary afferent neurons. (A) Expression of peripherin detected in DiI-labeled neurons. Representative photograph shows peripherin- immunoreactivity (IR) (green, FITC filter), DiI (red, cy3 filter), merged image, and differential interference contrast (DIC) image. DiI, a retrograde tracer, labeled maxillary trigeminal ganglionneurons with axons in the tooth pulp at 14 days after the DiI-labeling in the teeth. Arrows indicate peripherin-expressing neurons within DiI labeled population. Scale bar indicates 100 μm. (B) Pie chart shows population of peripherin-positive neurons within DiI-labeled trigeminal ganglion neurons.

RESULTS

Majority of DiI-labeled neurons was stained with peripherin

Retrograde transport fluorescent dye, DiI, placed in upper molars was detected as bright red dots in trigeminal ganglion sections. Dental primary afferent neurons were identified as a small subset of neurons that were stained with DiI (Fig. 1A). When upper molars were extracted and examined for confinement of DiI within dental pulp after the animals weresacrificed, DiI was observed only within the dental pulp, suggesting that unintended labeling of trigeminal ganglion neurons was minimal. As expected, DiI labeled neurons were detected only in the maxillary regions of the trigeminal ganglion (data not shown). The trigeminal ganglion was extensively stained with antibody against peripherin, a marker for small neurons. Out of 184±73 DiI-labeled neurons that were analyzed from 10 rats, average of 130±49 neuronsshowedimmunoreactivity to peripherin (72.3±8.5%), suggesting that the majority of dental primary afferent neurons were small-sized neurons (Fig. 1B).

A small subpopulation of tooth pulp neurons was stained with NF200

Myelinated population among dental primary afferent neurons was identified by the detection of neurofilament 200 (NF200), a marker of myelinated neurons. Fig. 2A shows that a significant portion of trigeminal ganglion neurons exhibited immunoreactivity to NF200. Compared with immunohistological result obtained with antibody against peripherin, NF200 positive neurons showed tendency of large diameter.Approximately 25.7±9.9% (n=54±34/197±71) amongDiI-labeled dentin primary afferent neurons showed immunoreactivity to NF200 (Fig. 2B).

Size distribution of peripherin or NF200 positive tooth pulp neurons

As expected, immunohistochemical analysis revealed preferential expression of peripherin in cytosol of small-sized neurons, whose size ranged between 100 μm^2 and 1,000 μm^2 (Fig. 3). Average area of peripherin positive neurons was 511.8±195 μm^2. NF200 was mostly detected in relatively larger cells, whose soma ranged between 700 μm^2 and 1,800 μm^2 with an average of 1205.4±274 μm^2 (Fig. 3). There are small number of neurons that were reacted to both antibodies against peripherin and NF200 (Supplementray Fig. 1A).

Dye placement surgery of tooth pulp did not induce neuronal damage

Since it is possible that DiI placement surgery performed in the upper molars might damage the dental primary afferent neurons innervated in the tooth pulp, and the injured neurons might show modified expression of cytoskeletons, expression of neuronal injury marker, cyclic AMP-dependent transcription factor 3 (ATF-3) was investigated in trigeminal ganglion to eliminate the

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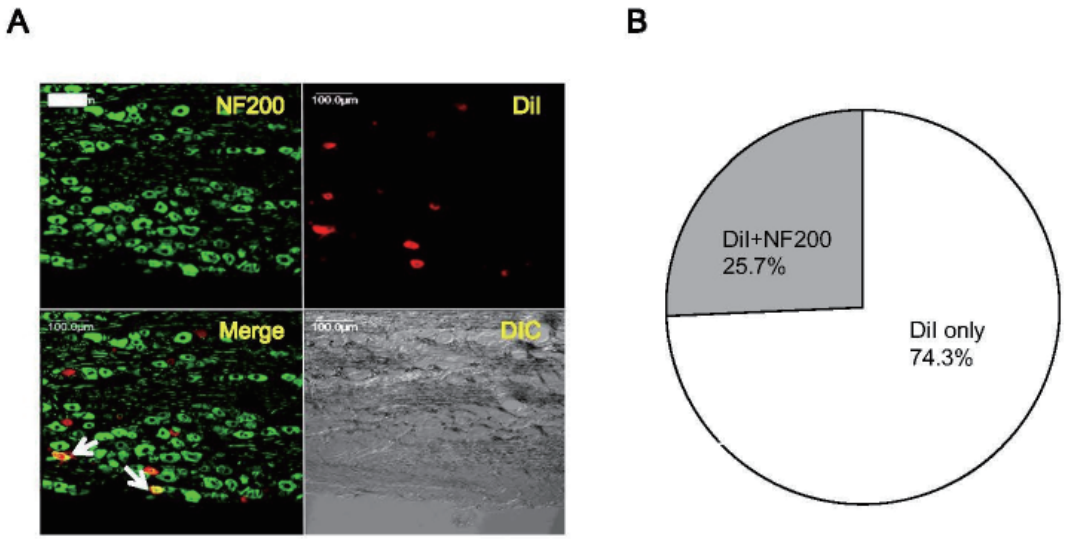

Fig. 2. Expression of neurofilament 200 in DiI labeled dental primary afferent neurons. (A) Expression of neurofilament 200 detected in DiI-labeled neurons. Representative photograph shows NF200-IR (green, FITC filter), DiI (red, cy3 filter), merged image, and differential interference contrast (DIC) image. Arrows indicate NF200 expressing neurons within DiI labeled population. Scale bar indicates 100 μm. (B) Pie chart shows population of NF200-positive neurons within DiI-labeled trigeminal ganglion neurons.

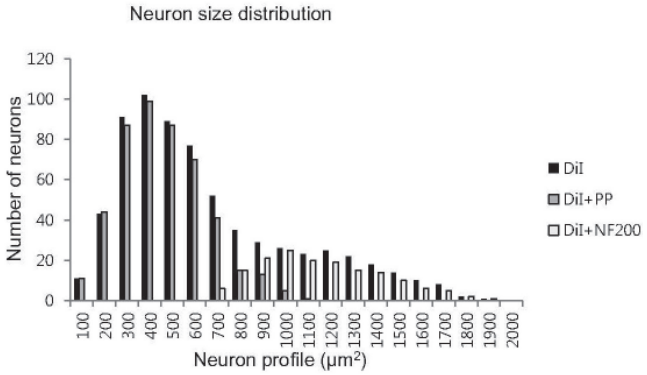

Fig. 3. Size distribution of peripherin or neurofilament 200 positive DiI-labeled neurons. Frequency histogram shows the distribution of peripherin or NF200 expressing neurons among DiI-labeled neurons by soma area. Peripherin was confined mainly in small neurons whereas neurofilament 200 was detected in population of large neurons.

possibility. Immunohistochemical analysis detected expression of ATF-3 only in 8.7±6.7% (n=16.8±13.0/215.7±93.3) of DiI-labeled dental primary afferent neurons (Fig. 4). In contrast, extensive expression of ATF-3 was observed in the mandibular region of trigeminal ganglion neurons from animals that had received transection injury in inferior alveolar nerve (Supplementary Fig. 1B).

DISCUSSION

In order to characterize nociceptive and non-nociceptive neurons, neurochemical properties of dental primary afferent neurons were determined by immunohistochemical analysis of retrogradely labeled trigeminal ganglion neurons in this study. Peripherin and NF200 [15-17] were used as markers for small neurons and myelinated neurons, respectively. As the results, 72.3±8.52% of labeled neurons exhibited characteristics of C-fiber-related nociceptive neurons, such as small size,expression of peripherin and lack of NF200. However, 25.7±9.9% of neurons wasstained with the antibody against NF200 and had large diameter, providing evidences of non-nociceptive population among dental primary afferent neurons. These results correlate well with our previous report that argued for similar proportion of non-nociceptive neurons among dental primary afferents based on electrophysiological observation [13].

Large population of small unmyelinated neurons suggests that detection of nociceptive signal is an important role of dental primary afferent neurons. Direct detection of nociceptive thermal stimuli by dental primary afferent neuron was suggested in our previous study by functional expression of temperature sensitive TRP channels in dental primary afferent neurons [14]. We have also reported expression of other nociceptive-related proteins, such as $P2X_2$, $P2X_3$ and $Na_V1.8$ [13]. Together with the results from current study, these observations suggest that detection of nociceptive stimuli is an important role of nociceptive dental primary afferent neurons.

It is noteworthy to find that significant subpopulation of dental primary afferent neurons showed characteristics of Aβ nerve

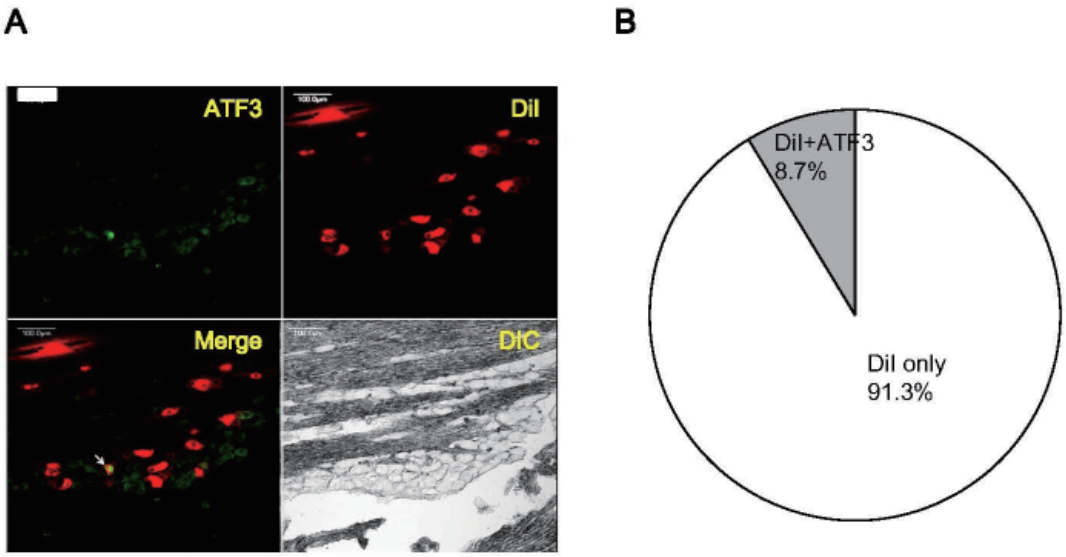

Fig. 4. Detection of neuronal damage was minimal in DiI-labeled neurons. (A) Neuronal damage caused by retrograde transport labeling of dental primary afferent neurons was minimal. Representative photograph shows scarce detection of ATF3-IR (green, FITC filer) among DiI (red, Cy3 filter) labeled trigeminal ganglion neurons. Scale bar indicates 100 μm. (B) Pie chart shows population of ATF3-IR neurons within DiI-labeled trigeminal ganglion neurons.

fibers. In contrast to the traditional view that dental primary afferent neurons are entirely consist of nociceptors, several lines of studies reported non-nociceptive Aβ nerve fibers in dental primary afferent neurons based on electron microscopic observation [2, 8, 22] and response of human subjects to weak electrical stimulation of tooth pulp [2, 7]. Since Aβ nerve fibers serve as non-nociceptive low-threshold mechanoreceptors and altered central processing of peripheral input from Aβ nerve fibers induces mechanical allodynia [23], it is possible that some of the NF200 positive neurons may be mechanosensitive Aβ afferent neurons that are responsible for detection of fluid movement within dentinal tubules,as proposed in hydrodynamic theory,and the altered central processing of information of the dentinal fluid movement might perceived as the dentin hypersensitivity. More sophisticated speculation on these Aβ dental primary afferent neurons is desirable for understanding of dentin hypersensitivity, along with biomechanical investigation on fluid dynamics within the dentinal tubules.

Several literatures suggested that over 60% of axons of inferior alveolar nerve neurons were myelinated [24-27], which showed discrepancyto our data in whichonly 25.7% were myelinated. In addition, more recent study reported that most of parental axons traced with horseradish peroxidase placed in upper molars of rats were myelinated [28]. Investigation of conduction velocity of pulp neurons suggested demyelination of myelinated nerve fibers upon entrance to pulp cavity [29], which was supported by electron microscopic investigationwhich observed 33.5% of myelination in tooth pulp neurons in contrast to more than 59% of myelination in inferior alveolar nerve neurons in rats [25]. However, this might be not the case in our experimental setup, since immunofluorescence of NF200 was investigated in the trigeminal ganglion where the somas of the pulp neurons were located. One possible explanation for this discrepancy is that incidence ofmyelination in electron microscopic studies were high because Aβ as well as Aδ fibers are detected in electromicroscopic studies, whereas in our immunohistochemical analysis, immunofluorescent intensity of NF200 from thinly myelinated Aδ population was too weak to be detected as positive¸ and only thickly myelinated Aβ fiber neurons were counted. In line with this, the diameters of NF200 positive neurons were distinct fromdiameters of peripherin positive neurons, suggesting that detection of small myelinated neurons by NF200 was difficult in our experimental setup.

It is possible that placement surgery of retrograde transport fluorescent dye in tooth pulp might cause damage and subsequent plastic change to the dental primary afferent neurons. If this is the case, the results obtained from the labeled neurons might not represent physiologic state. However, limited detection of neuronal injury marker ATF3 in retrogradely labeled neurons suggests that our surgical protocols did not damage the tooth pulp neurons significantly and changes in neurofilaments expression was minimal.

In conclusion, while majority of tooth pulp neurons showed anatomical and immunohistochemical characteristics of nociceptive neurons, significant subpopulation was detected as

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non-nociceptive large myelinated neurons, which might serve as mechanotransducers that detect movement of dentinal fluid. It is possible that altered processing of the information from these non-nociceptive large myelinated neuronsmight contribute to the generation of dentin hypersensitivity, in which low-threshold mechanical stimulation could be perceived as nociception.

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