Vesicular Glutamate Transporters in Axons That Innervate the Human Dental Pulp

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Abstract

Introduction: Vesicular glutamate transporters (VGLUTs) are involved in the transport of transmitter glutamate into synaptic vesicles and are used as markers for glutamatergic neurons. **Methods:** To assess which types of VGLUTs are involved in the glutamate signaling in pulpal axons and to investigate their distribution, we performed light microscopic immunohistochemistry by using antibodies against VGLUT1, VGLUT2, calcitonin gene-related peptide, and Western blot analysis in human dental pulp. **Results:** VGLUT1 was expressed in a large number of pulpal axons, especially in the peripheral pulp where the axons branch extensively. The VGLUT1 immunopositive axons showed bead-like appearance, and the majority of these also expressed calcitonin gene-related peptide. VGLUT2 was expressed in few axons throughout the pulp. **Conclusions:** Our findings suggest that VGLUT1 is involved mainly in the glutamate-mediated signaling of pain, primarily at the level of the peripheral pulp. *(J Endod 2012;38:470–474)*

Key Words

Dental pulp, glutamate, immunohistochemistry, pain, vesicular glutamate transporter

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Glutamate, the major excitatory neurotransmitter in the central nervous system (CNS), is transported into vesicles by a family of vesicular glutamate transporters (VGLUTs) before it can be released by stimulation. Most glutamatergic neurons express 2 of the 3 VGLUTs that have been described so far, VGLUT1 and VGLUT2, which makes these transporters useful markers for glutamatergic neurons (1, 2). Studies that use immunohistochemistry and *in situ* hybridization have shown that VGLUT1 and VGLUT2 are segregated and complementary in many CNS regions and are expressed by distinct subpopulation of primary sensory afferents. Whereas VGLUT1 is predominantly expressed by low-threshold mechanoreceptive and proprioceptive afferents, VGLUT2 is predominantly expressed by nociceptive afferents (3–6). VGLUT1 and VGLUT2 are also expressed differently in peripheral sensory afferents to different organs; sensory afferents from muscle spindles express VGLUT1 but not VGLUT2 (7, 8), whereas those from the gut express VGLUT2 but not VGLUT1 (9). These distinct expression patterns possibly reflect differences in the sensory processing by glutamatergic afferents innervating various peripheral organs.

The dental pulp is densely innervated by nociceptive afferents (10–13) and is thus used frequently as a model system to study pain mechanisms. Glutamate signaling plays a crucial role in pain transduction (14, 15). Jackson and Hargreaves (16) have shown that superfusion of bovine dental pulp with glutamate causes release of the nociceptive neuropeptide calcitonin gene-related peptide (CGRP), and that application of capsaicin to the dental pulp, which causes acute burning pain, evokes release of glutamate through activation of the capsaicin receptor TRPV1. This suggests that glutamate can both be released from and activate peptidergic axons in the dental pulp. However, currently little is known about (1) which types of VGLUTs are involved in the glutamate signaling and (2) the distribution pattern of immunoreactivity for VGLUTs in the dental pulp. To address these issues, we investigated the expression of VGLUT1, VGLUT2, and CGRP in the axons of human dental pulp by using light microscopic immunohistochemistry.

Materials and Methods

Tissue Preparation

Healthy human dental pulps of first and second premolars, which were extracted during the course of orthodontic treatment from 17- to 32-year-old patients at the Department of Oral Surgery, Kyungpook National University Hospital, South Korea, were used in this study. The informed consent of all human subjects who participated in the experimental investigation reported or described in this article was obtained after the nature of the procedure and possible discomforts and risks had been fully explained. Ethical approval for the study was obtained in conformity with the policy of the Research and Ethics Committee of Kyungpook National University. After extraction, the hard tissues of the teeth were cut with a diamond bur, and the pulps were carefully removed. Seventeen pulps from 11 patients were used in this study, 11 pulps for immunofluorescent staining and 6 pulps for Western blots.

Immunofluorescent Staining

The pulps were fixed with 4% (w/v) paraformaldehyde in 0.1 mol/L phosphate buffer (PB) (pH 7.4) for 2 hours and cryoprotected in 30% sucrose in PB overnight at 4°C. The next day, 30-μm-thick sections were cut on a freezing microtome, washed with phosphate-buffered saline (PBS) (0.01 mol/L, pH 7.4), and treated with 50%

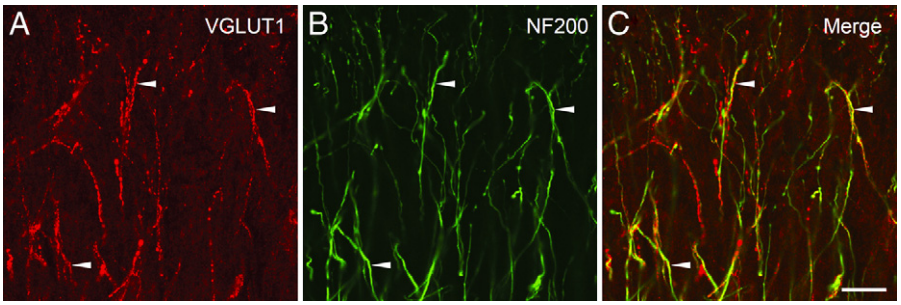

Figure 1. Double immunofluorescent staining for VGLUT1 and NF200 (a marker for neuron) in coronal portion of human dental pulp. (*A–C*) VGLUT1-immunopositive axons co-stained for NF200 (*arrowheads*), indicating VGLUT1 was expressed in pulpal axons. Scale bar = 20 μm.

ethanol for 30 minutes to enhance penetration and with 10% normal donkey serum (Jackson ImmunoResearch, West Grove, PA) for 30 minutes to mask secondary antibody binding sites. The sections were incubated overnight in 1 or 2 primary antibodies in PBS, guinea pig anti-VGLUT1 (#135304), rabbit anti-VGLUT2 (#135402; both from Synaptic Systems, Goettingen, Germany; 1:3000), mouse anti-neurofilament 200 (NF200, N52, #N0142; Sigma, St Louis, MO; 1:10,000), or rabbit anti-CGRP (#24112; ImmunoStar Inc, Hudson, WI; 1:1000), washed with PBS, and incubated for 3 hours with fluorescein isothiocyanate– or Cy3-conjugated secondary antibodies raised in donkey (Jackson ImmunoResearch; 1:200). The sections were then mounted on slides and coverslipped with Vectashield (Vector, Burlingame, CA), and micrographs were obtained with an Exi digital camera (Q-Imaging Inc, Surrey, CA) attached to a Zeiss Axioplan 2 conventional fluorescence microscope or with a Zeiss LSM510META laser confocal microscope (Carl Zeiss Inc, Jena, Germany). To control for specificity of the antibodies, sections were processed as described above, except that primary or secondary antibodies were omitted: Omission of primary or secondary antibodies eliminated specific staining. Preadsorption with blocking peptides for VGLUT1 (#135-3P) and VGLUT2 (#135-40P; both from Synaptic Systems; 15 μg/mL) also completely abolished the respective staining.

Analysis for Immunopositive Axons

For quantitative analysis for the density of VGLUT1- and VGLUT2-immunopositive axons in human dental pulp, total of 27 images were captured from 3 sections of each of 3 pulps with Exi digital camera (Q-Imaging Inc). The images were taken by using $\times$40 objectives (1360 $\times$ 1036 pixels) in the central and peripheral portions of the coronal pulps. The threshold level for defining immunopositive axons was determined at 100–120 gray levels by using ImageJ software from images with 256 gray levels. Area fractions of VGLUT1- and VGLUT2-immunopositive axons were analyzed. Difference in density of VGLUT1- and VGLUT2-immunopositive axons was analyzed with unpaired Student's *t* test ($P < .01$). For analysis of co-localization of VGLUT1 and CGRP, 3 z-series images from each of 9 sections from 3 pulps were prepared in the coronal and radicular pulp with C-Apochromat 40$\times$/1.2 NA objectives (1 μm of optical slice thickness) on a confocal microscope (LSM 510 META; Carl Zeiss Inc). Co-localization was evaluated by counting the number of identifiable single axons showing immunoreactivity for VGLUT1 alone and for both VGLUT1 and CGRP from the z-series images.

Western Blot Analysis

Human dental pulps or rat brainstem at the level of trigeminal caudal nucleus receiving orofacial afferents were removed and homogenized with a plastic pestle in extraction buffer containing 20 mmol/L Tris-HCl (pH 7.4), 5 mmol/L ethylenediaminetetraacetic acid, 140 mmol/L NaCl, 1% Triton X-100, 0.1% sodium dodecylsulfate, 1 mmol/L sodium orthovanadate (Na_3VO_4), 1 mmol/L phenylmethylsulfonyl fluoride, 10 mmol/L sodium fluoride, and 1 μg/mL aprotinin. The extracts were centrifuged at 12,000*g* for 20 minutes at 4°C, and protein content in the supernatant was measured by using a Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA). The protein lysates were denatured and applied on a sodium dodecylsulfate polyacrylamide gel for electrophoresis. Proteins were transferred to Immobilon-P membranes (Millipore Co, Bedford, MA) that were soaked in blocking solution and incubated overnight at 4°C with the primary antibodies guinea pig anti-VGLUT1 (1:1000; Synaptic Systems Inc), rabbit anti-VGLUT2 (1:1000; Synaptic Systems Inc), and mouse

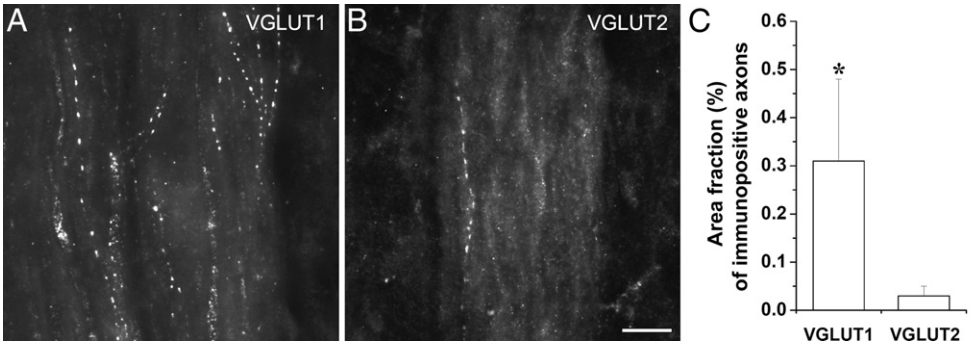

Figure 2. Immunofluorescent staining for VGLUT1 (*A*) and VGLUT2 (*B*) in central portion of human coronal pulp, and a histogram (*C*) showing area fraction (%) of VGLUT1 and VGLUT2 immunopositive axons in coronal portion of human dental pulp. (*A* and *B*) VGLUT1 was observed in large number of pulpal axons in contrast to VGLUT2, which was observed in few axons. (*C*) Area fraction (%) of VGLUT1-immunopositive axons was significantly higher than that of VGLUT2-immunopositive axons (unpaired *t* test, $P < .01$). Scale bar = 20 μm.