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EXPRESSION OF VESICULAR GLUTAMATE TRANSPORTERS IN TRANSIENT RECEPTOR POTENTIAL MELASTATIN 8 (TRPM8)-POSITIVE DENTAL AFFERENTS IN THE MOUSE

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Abstract—Transient receptor potential melastatin 8 (TRPM8) is activated by innocuous cool and noxious cold and plays a crucial role in cold-induced acute pain and pain hypersensitivity. To help understand the mechanism of TRPM8-mediated cold perception under normal and pathologic conditions, we used light microscopic immunohistochemistry and Western blot analysis in mice expressing a genetically encoded axonal tracer in TRPM8-positive (+) neurons. We investigated the coexpression of TRPM8 and vesicular glutamate transporter 1 (VGLUT1) and VGLUT2 in the trigeminal ganglion (TG) and the dental pulp before and after inducing pulpal inflammation. Many TRPM8+ neurons in the TG and axons in the dental pulp expressed VGLUT2, while none expressed VGLUT1. TRPM8+ axons were dense in the pulp horn and peripheral pulp and also frequently observed in the dentinal tubules. Following pulpal inflammation, the proportion of VGLUT2+ and of VGLUT2+/TRPM8+ neurons increased significantly, whereas that of TRPM8+ neurons remained unchanged. Our findings suggest the existence of VGLUT2 (but not VGLUT1)-mediated glutamate signaling in TRPM8+ neurons possibly underlying the cold-induced acute pain and hypersensitivity to cold following pulpal inflammation.

Key words: TRPM8, vesicular glutamate transporter, inflammation, dental pulp, cold pain.

INTRODUCTION

Transient receptor potential melastatin 8 (TRPM8), a member of TRP channel superfamily, is activated by innocuous cool and noxious cold, as well as by menthol (Dhaka et al., 2007; Knowlton and McKemy, 2011). It plays a crucial role in mediating cold-induced acute pain and hypersensitivity following inflammation and nerve injury (McKemy et al., 2002; Bautista et al., 2007; Knowlton et al., 2010). Stimulation of TRPM8 in the central afferents of dorsal root ganglion (DRG) neurons by cold or menthol increases the frequency of miniature excitatory postsynaptic currents in the postsynaptic dorsal horn neurons, suggesting that transmission of TRPM8-mediated cold in the 1st relay nucleus in the CNS is mediated by glutamate (Baccei et al., 2003; Tsuzuki et al., 2004; Wrigley et al., 2009). Studies with electrical and chemical stimulation of peripheral axons, and administration of glutamate into peripheral tissues, also showed that glutamate released from peripheral axons plays a crucial role in the signaling of acute pain and hyperalgesia (Du et al., 2001; Svensson et al., 2003; Miller et al., 2011). However, the type of vesicular glutamate transporters (VGLUTs) associated with the signal transduction of TRPM8-meidated cold remains unknown.

VGLUTs are involved in the packing of glutamate into synaptic vesicles prior to exocytotic release and thus play a crucial role in the glutamatergic transmission (Fremeau et al., 2004). Of the three VGLUTs, VGLUT1 and VGLUT2 are expressed by largely non-overlapping and functionally distinct populations of primary sensory neurons (Fremeau et al., 2004; Brumovsky et al., 2007). Recent reports, including studies using VGLUT2-deficient mice, suggest that VGLUT2-dependant glutamate release from primary sensory afferents plays an important role in normal acute nociception and pathologic pain (Moechars et al., 2006; Leo et al., 2009; Liu et al., 2010; Scherrer et al., 2010; Lagerstrom et al., 2011; Rogoz et al., 2012). Specifically, VGLUT2 increases in the trigeminal ganglion (TG) neurons following Complete Freund's Adjuvant (CFA)-induced pulpal inflammation, suggesting that VGLUT2-dependant glutamate signaling may mediate pulpal inflammatory pain (Yang et al., 2014). However, little is known about the alteration in expression of TRPM8 and the associated VGLUTs in TG neurons following dental pulp inflammation.

To help understand the role of neuronal TRPM8 and VGLUT in the dental hypersensitivity to cold, we

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Abbreviations: CGRP, Calcitonin Gene-Related Peptide; CFA, Complete Freund's Adjuvant; Cy, Cyanine; DRG, dorsal root ganglion; EDTA, ethylenediaminetetraacetic acid; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; LM, light microscopy; NDS, normal donkey serum; NF200, neurofilament 200; PB, phosphate buffer; PGP9.5, protein gene product 9.5; TG, trigeminal ganglion; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline; TMR-DA, tetramethylrhodamine-conjugated dextran amine; TRPM8, transient receptor potential melastatin 8; VGLUT, vesicular glutamate transporter.

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investigated the coexpression of TRPM8 and VGLUTs in the TG and dental pulp before and after CFA-induced pulpal inflammation, using immunohistochemistry and Western blot analysis in transgenic mice expressing a genetically encoded axonal tracer in TRPM8-positive neurons.

EXPERIMENTAL PROCEDURES

Animals and tissue preparation

All experimental procedures and animal care were conducted in accordance with the National Institutes of Health guidelines and were approved by the Kyungpook National University Intramural Animal Care and Use Committee (permit number, KNU 2011-4). Experiments were designed to minimize the number of animals used and their discomfort. Transgenic ($TRPM8^{GFP}$) mice, expressing enhanced green fluorescent protein (GFP) by the TRPM8 transcriptional promoter were generated, mated to C57BL/6 mice, and their offspring were genotyped as described previously (Takashima et al., 2007). Twenty-nine male $TRPM8^{GFP}$ mice (weight 25–30 g), aged 6–10 weeks, were used for this study: Nineteen for light microscopic (LM) immunohistochemistry (three for normal group, five each for CFA treatment with 3 days of survival and their control, and six for retrograde labeling of tooth pulp afferents), and 10 for Western blotting.

Tooth pulp inflammation model and retrograde labeling

To induce tooth pulp inflammation, mice were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and the occlusal enamel and dentin of the right maxillary 1st (M1) and 2nd (M2) molars were filed off to just before exposing the pulp using a low-speed dental drill with a round bur under water-cooling. A fine paper point soaked in 50% CFA solution in saline (experimental) or saline for control (sham) was applied to the exposed dentinal surfaces for 5 min. For retrograde labeling of pulpal afferents, the dental pulp of M1 and M2 was exposed and crystals of tetramethylrhodamine-conjugated dextran amine (TMR-DA, 3000 MW, D3308, Invitrogen, Carlsbad, CA, USA) were applied for 5 min. After that, the dentinal or pulpal surfaces were sealed with dental cement. Mice were euthanized 3 days after the application of CFA, saline or TMR-DA.

LM immunohistochemistry

For immunofluorescence, mice were deeply anesthetized with sodium pentobarbital (40 mg/kg, i.p.), transcardially perfused with heparinized normal saline, followed by freshly prepared fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The right TGs and maxillae including M1 and M2 were dissected out and postfixed in the same fixative for 2 h at 4 °C. For immunofluorescence of the dental pulp, the fixed maxillae were decalcified in 10% ethylenediaminetetraacetic acid (EDTA, pH 7.4) at 4 °C for 6 days, changing the EDTA solution daily, and then were rinsed in PB for 30 min. Samples were cryoprotected in 30% sucrose in PB overnight at 4 °C. The next day, 40-μm-thick sections were cut on a freezing microtome.

Sections of TGs and dental pulp were permeabilized with 50% ethanol for 30 min, blocked with 10% normal donkey serum (NDS, Jackson ImmunoResearch, West Groove, PA, USA) for 30 min, and incubated overnight in a mixture of rabbit anti-GFP antibody and VGLUT1, VGLUT2, protein gene product 9.5 (PGP9.5), Calcitonin Gene-Related Peptide (CGRP) or anti-neurofilament 200 (NF200) antibodies. The primary antibodies were rabbit anti-GFP (1:3000, A11122, Invitrogen, Carlsbad, CA, USA), guinea-pig anti-VGLUT1 (1:2000, 135304, Synaptic Systems, Gottingen, Germany), guinea-pig anti-VGLUT2 (1:1000, Af670, Frontier Institute Co. Ltd., Hokkaido, Japan), mouse anti-PGP 9.5 (1:5000, YM8104, Accurate Chemical and Scientific Corp., Westbury, NY, USA), mouse anti-CGRP (1:1000, ab81887, Abcam, Cambridge, MA, USA) or mouse anti-NF200 (1:100,000, N0142, Sigma–Aldrich, St. Louis, MO, USA). On the next day, the sections were rinsed with phosphate-buffered saline (PBS; 0.01 M, pH 7.4) several times and incubated for 3 h in a mixture of fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit and Cyanine 3 (Cy3)-conjugated donkey anti-guinea-pig or anti-mouse antibody or in a mixture of FITC-conjugated donkey anti-rabbit and Cy5-conjugated donkey anti-guinea-pig or donkey anti-mouse antibodies (1:200, Jackson ImmunoResearch). The sections were then rinsed, mounted on slides, and coverslipped with Vectashield (Vector Lab, Burlingame, CA, USA). Micrographs were obtained with an Exi digital camera (Q-imaging Inc., Surrey, CA, USA), attached to a Zeiss Axioplan 2 fluorescence microscope (Carl Zeiss Inc., Jena, Germany) or with a confocal microscope (LSM 510 META, Carl Zeiss, confocal images were acquired at a same optical slice thickness for all channels).

To confirm that the inflammation is confined to the dental pulp, sections containing dental pulp were processed, as described above, using goat anti-CD64 antibody (1:500, sc-7642, Santa Cruz Biotechnology, Dallas, TX, USA), and FITC-conjugated donkey anti-goat antibody (1:200, Jackson ImmunoResearch), as primary and secondary antibodies, respectively.

To control for the specificity of the primary antibodies, sections were processed as described above, except that primary or secondary antibodies were omitted or blocking peptides were added: Omission of primary or secondary antibodies or preadsorption with blocking peptides for VGLUT1 (15 μg/ml, 135-3P, Synaptic Systems) and VGLUT2 (10 μg/ml, G07B, Frontier Institute Co. Ltd.) abolished the respective staining. The specificity of the anti-GFP antibody was confirmed in a non-GFP-expressing mouse line (Moldrich et al., 2010): GFP was not detected in the TG and dental pulp in the wild-type C57BL/6 mice using a rabbit anti-GFP antibody.

Quantitative analysis

To quantify the proportion of TRPM8+, VGLUT1+, and VGLUT2+ somata in the TG, a total of 36–48 images from the maxillary portion of the TG, which contains