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치수 구심성 신경에서 기계자극민감성 이온 채널의 발현 분석

Characterization of Mechanosensitive Channels in Dental Primary Afferent System

2017 년 8 월

서울대학교 대학원

치의과학과 신경생물학 전공

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Characterization of Mechanosensitive Channels in Dental Primary Afferent System

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ABSTRACT

Characterization of Mechanosensitive Channels in Dental Primary Afferent System

Hue Vang

Dentin hypersensitivity is the acute, severe pain evoked by subtle stimulation on exposed dentin. This paradoxical pain has raised the need to identify the molecular and functional expression of mechanically sensitive ion channels in the dental pulp. Piezo ion channels are the mechanosensitive ion channels expressed in neurons and various cells of internal structure for sensation and regulation. Here I examined the molecular and functional expression of Piezo ion channels in dental primary afferent system by using RT-PCR, single-cell RT-PCR, in situ hybridization and whole cell patch clamp. Piezo1 mRNA was detected in whole trigeminal ganglion and pulp tissue by RT-PCR. However Piezo1 was not detected by single-cell RT-PCR in DiI-labeled dental primary afferent (DPA) neurons nor in odontoblasts. When odontoblasts and DPA neurons were examined for Piezo2, Piezo2 mRNA was not detected in odontoblasts, but were detected in DPA neurons. DPA neurons with Piezo2 mRNA or Piezo2-like currents were further characterized based on their neurochemical and electrophysiological properties. Piezo2 mRNA was found mostly in medium- to large-sized DPA neurons, with the majority of these neurons also positive for Nav1.8, CGRP and NF200, whereas only a minor

population were positive for TRPV1, IB4 and peripherin. Whole cell patch clamp recordings revealed Piezo2-like, rapidly inactivating currents evoked by mechanical stimulation in a subpopulation of DPA neurons. Rapidly inactivating currents were pharmacologically blocked by ruthenium red, a compound known to block Piezo2 and were also reduced by siRNA-mediated Piezo2 knockdown. Piezo2-like currents were observed almost exclusively in IB4-negative DPA neurons, with the current amplitude larger in capsaicin-insensitive DPA neurons than the capsaicin-sensitive population. My findings show that subpopulation of DPA neurons are indeed mechanically sensitive. Within this subpopulation of mechanosensitive DPA neurons, I have identified the Piezo2 ion channel as a potential transducer for mechanical stimuli, contributing to rapidly inactivating inward currents. Piezo2-positive DPA neurons were characterized as medium to large-sized neurons with myelinated A-fibers, containing nociceptive peptidergic neurotransmitters.

Key Words: Dental primary afferent, Dental pulp, Odontoblast, Mechanosensitive ion channel, Piezo1, Piezo2.

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BACKGROUND

1. Tooth pain

Tooth is the hardest tissue in mammals, constructed by minerals matrix to form of enamel, dentin covering inside soft tissue (tooth pulp). In the tooth pulp comprises cellular soft tissue, circulation system, lymphatic system and nerve supply. The nerve terminals are extended to the dentinal tubules and closely arranged with odontoblasts at the outer layer of pulp. Odontoblasts act a role of dentinal formation in tooth development or dentin recovery from pathological state to protect the tooth pulp (Charadram et al. 2012; Thivichon-Prince et al. 2009). As well, odontoblasts may serve as a sensor to transduce sensory signal to the nerve terminal. Nerve supplies in the tooth pulp consist sensory axons and sympathetic axons (Beasley and Holland 1978; Pimenidis and Hinds 1977; Tonder and Naess 1978; Weiss et al. 1972). The sensory axons in mammals tooth have a role in physiological sense (Abd-Elmeguid and Yu 2009; Byers 1984; Hildebrand et al.

1995). Dental pain is different from other somatosensory pain, as we feel exaggerated pain such as dentinal hypersensitivity when the sense organs in the tooth come into contact with its external stimuli. This exaggerated pain usually leads to the need for immediate clinical treatment. Through the long lasting studies three hypotheses have been described (Fig. 1) (Chung, Jung and Oh 2013; Dowell and Addy 1983; West 2006). Each hypothesis explains the dentinal hypersensitivity by distinct molecular biological mechanisms.

Neural theory: One of nociceptive sensitivity mechanism of tooth is that the stimulation from outside of tooth such as heat, cold, hypertonic substance of chemical substances directly activates the nerve terminal in the dentinal tubules by the specific receptors present in nerve terminal. This leads to membrane potential change or action potential signaling to cell body in trigeminal ganglion (dental primary afferent neuron) and the central nervous system. Immunohistochemical staining, single-cell RT-PCR and electrophysiological recording have been showed nociceptive TRP channels such as TRPV1, TRPV2, TRPA1, TRPM8 expression in dental primary afferent (DPA) neurons (Chung, Jung and Oh 2013; Gibbs, Melnyk and Basbaum 2011; Hermanstyne et al. 2008; Kim et al. 2011; Park et al. 2006). The expression of nociceptive TRP channels may explain mechanism of tooth sensitivity during temperature changes in the mouth. TRPV1 channel activates when temperature increase up to 43˚C and TRPV2 are sensitive to heat more than 53˚C (Bender et al. 2005; Caterina et al. 1999), however cold sensitivity is evoked by TPPM8 and TRPA1 (Kim et al. 2011; Park et al. 2006).

Odontoblasts transducer theory: Odontoblasts are surrounding outer layer of pulp in structure and extend their processes into dentinal tubules and floating in dentinal tubule fluid. Odontoblasts have been suggested as sensory transducing cells by transmitting signals to nerve ending via neurotransmitters. The evidences to support the role of odontoblast transducer showed TRP channels expression in neonate rat and human odontoblasts such as TRPV1, TRPV2, TRPV3, TRPV4 and TRPM3 by single cell RT-PCR, immunohistochemistry and calcium imaging (Egbuniwe et al. 2014; Son et al. 2009). However, in previous studies with isolated odontoblasts from adult rat, TRPV1 and TRPV2 were not detected by single cell RT-PCR (Yeon et al. 2009). On the other hand human odontoblasts express TRPV1, TRPM8 and TRPA1 (El Karim et al. 2011). Therefore, odontoblasts transducer theory still is controversial to explain noxious thermal sensitivity. In other, odontoblast may serve in mechanical transducers as well as expression of mechanosensitive K^+ channels and N-type Ca^{+2} channels (Allard et al. 2000; Magloire et al. 2009).

Odontoblasts are the excitable cells. Odontoblast may be activated by a specific stimuli to mediate tooth pain (Magloire et al. 2009). Recent studies have shown that external stimulation evoked odontblasts to release neurochemical substances such as ATP (Fu et al. 2015; Sato et al. 2015; Shibukawa et al. 2015) to induce activation of purinergic $P2X_3$ receptors in nearby dental primary afferent terminals (Alavi, Dubyak and Burnstock 2001). The activation of TRP channels, TRPA1 and TRPV1 in human odontoblasts induces ATP release (Egbuniwe et al. 2014; Shibukawa et al.

2015). Therefore, odontoblasts may be responsible for dental sensory function (Chung, Jung and Oh 2013; Lin et al. 2014).

Hydrodynamic theory: The structure of dentin pulp complex shows that A-fibers terminal (Byers and Narhi 1999) and odontoblast processes are arranged within the dentinal tubules. While the dentinal tubule fluid flow by external stimulation led a mechanical stimuli to activate the specific receptors on nerve terminal and odontoblast processes in dentinal tubule. The changing of outside tooth environment such as heat, cold, air puff and touching results in the rapid dentinal fluid movement in outward or inward direction, causing the mechanical force to directly stimulate the mechanoreceptors in dental primary afferent terminal or odontoblasts. The previous study showed the putative mechanosensitive ion channels in dental primary afferent neurons, but most of these ion channels are established for chemical and thermosensitive function rather than mechanoreceptors. However, no strong evidences have been found for mechanoreceptor expression in dental afferent system to support the hypothesis (Absi, Addy and Adams 1987; Chung, Jung and Oh 2013; Lin et al. 2014).

The mentioned hypotheses have been limited to understand overall of tooth sensation, and the critical molecular mechanism are yet uncovered. Up to date, the dentinal hypersensitivity is still puzzling at the moment and needs to be answered to clarify the mechanism.

2. Mechanical sensitivity

The somatosensory system is fundamental for physiological sensation in mammals. The sensory modalities are divided in proprioception (sense of positioning or balance), mechanoreception (sense on touch, pressure) and nociception (pain, chemical, temperature). Mechanoreception is the response to mechanical stimulation for sense of touch, hearing and pain (allodynia). It allows the tactile system to respond to various stimuli via dorsal horn ganglion and trigeminal ganglion neurons. The cutaneous mechanoreceptors on the skin mostly are myelinated Aβ-fibers, some of thinly myelinated Aδ-fibers, and unmyelinated C-fibers. In vitro studies have described mechanosensitive channels in somatosensory neurons and the receptive field of the skin.

The mechanosensitive ion channels in sensory neurons found up to date are TRP channels or other cation channels. In mammalian, the TRP family channels are divided into six or seven families: TRPC (Canonical), TRPM (Melastatin), TRPV (Vanilloid), TRPA (Ankyrin), TRPP (Polycystin), TRPML (Mucolipin) and TRPN (nompc) (Chung and Oh 2009; Clapham 2003; Nilius and Honore 2012). The TRPC1, TRPC6, TRPV1, TRPV2, TRPV4, TRPM3, TRPM7, TRPA1 and TRPP2 channels were established as putative mechanosensitive TRP channels in neurons (Chung and Oh 2009). Other cation channels such as potassium channels TREK-1 (Fink et al. 1996; Maingret et al. 2000), TREK-2 (Bang, Kim and Kim 2000), TRAAK (Fink et al. 1998; Noel et al. 2009; Yamamoto, Hatakeyama and Taniguchi 2009) and mechanosensitive sodium channels DEG/ ENaC family (Chatzigeorgiou

et al. 2010; Eastwood and Goodman 2012) have also been found to have mechanosensitive properties. Most of putative mechanical ion channels mentioned above were found in sensory neurons and detected the stretch activation mechanosensitivity in vitro (Chung and Oh 2009). In dental physiology, TRP channels such as TRPV1, TRPV2, TRPA1 and TRPM8 or ASIC3 have been discussed as putative mechanosensitive ion channels to have a role in part of dental sensitivity (Hermanstyne et al. 2008; Ichikawa and Sugimoto 2000; Kim et al. 2011; Park et al. 2006).

On the skin, the perception of mechanical sensations depends on activation of two main types of mechanosensitive primary afferent neurons: first is the low threshold mechanoreceptive (LTM) neurons that respond to non-noxious (innocuous), low intensity, normally non-painful stimuli; and second is the high-threshold mechanoreceptive (HTM) neurons (nociceptors) that respond to noxious, high intensity, normally harmful/painful mechanical stimuli. LTMs and HTMs are also classified, according to the conduction velocities (CVs) of their nerve fibers, into Aβ-, Aδ- and C-fiber neurons. Both LTMs and HTMs conduct in all conduction velocity ranges (Aβ-, Aδ- and C-fiber), but most of the fast conducting myelinated Aβ-fiber neurons are LTMs, and most of the slowly conducting thinly myelinated Aδ-fiber and unmyelinated C-fiber neurons are nociceptors. Thus the hairy skin is innervated by Aβ-, Aδ- and C-fiber LTMs as well as HTMs including Aδ-HTMs (Djouhri 2016). The free nerve ending of thinly myelinated Aδ-fibers, and unmyelinated C-fibers that locate in epidermis/dermis of glabrous skin are noxious

high-threshold mechanoreceptors that respond to pain and thermal sensation (Abraira and Ginty 2013; Delmas, Hao and Rodat-Despoix 2011). The low-threshold mechanoreceptors locate in hairy skin and on other layers of glabrous skin such basal layer of epidermis, dermal papillae, deep dermis most innervated in myelinated Aβ-fibers to transduce the innocuous stimulation touching, pressure, vibrations. The mechanosensitive ion channels are classified by inactivation of receptors. Rapidly inactivating (RA) has the time constant of (τ) $\leq$ 10 ms, intermediate inactivating (IA) has the time constant (τ) between 10 – 20 ms and slowly inactivating (SA)) has the time constant (τ) more than 20ms (Coste et al. 2010; Hu and Lewin 2006; Smith and Lewin 2009).

The low-threshold mechanoreceptors (LTMRs) in mammals are prescribed of primary somatosensory neurons that function to sense external innocuous mechanical force. Generally LTMRs are composed of Aβ-LTMRs, Aδ-LTMRs and C-LTMRs, which distinguish in molecular, physiological, anatomical, and functional in characterization. The mechanosensitivity mechanisms of each low-threshold mechanoreceptors (LTMRs) are different based on the adapting, conduction velocity, anatomical structures. The low-threshold mechanoreceptors in cutaneous with specialized receptive field known on the hairy skin include Merkel cells, circumferental endings, and lanceolate endings (Bai et al. 2015; Li et al. 2011; Roudaut et al. 2012). In glabrous skin, there are Pacinian corpuscle, Meissner's corpuscle, Merkel cells, and Ruffini ending. (Abraira and Ginty 2013; Delmas, Hao and Rodat-Despoix 2011).

Pacinian corpuscles are the largest mechanoreceptors located in dermis of the mammals skin. Pacinian are innervated by large myelinated Aβ-fibers that transduce the mechanical stimuli in response to vibration (Ferrington, Nail and Rowe 1977). Pacinain corpuscles are rapidly adapting (RA2-LTMRs) (Iggo and Ogawa 1977).

Meissner's corpuscles are located in dermal papillae of glabrous skin and they are innervated by RA1-LTMRs. These receptive fields are respond to the skin motion, slip detection, and low frequency of vibration (20-40 Hz) (Johnson 2001).

Merkel cells first depicted by Friedrich Sigmund Merkel in 1875. They are mechanoreceptors in the mammals skin, innervated by myelinated fibers locate between the epidermis and dermis found both hairy and glabrous skin. In physiological recording, Merkel cells are slowly adapting type I (SA1), Aβ-SA1-LTMRs (Iggo and Muir 1969), which functioning as mechanically sensitive, excitatory and modulatory cells (Haeberle et al. 2004).

Ruffini endings are amplified dendritic endings with lengthened capsules that located in the dermis of glabrous and hairy skin, as well as in the periodontal ligament (Maeda et al. 1989). They are associated with SA2-LTMRs respond to the skin stretch (Johnson 2001).

Aβ RA-LTMRs lanceolate endings affiliate with guard hairs and awl/auchene hair follicles Aβ-LTMRs, Aδ-LTMRs and C-low threshold fibers are finely sensitive to hair deflection, light brush and touch (Abraira and Ginty 2013; Li et al. 2011; Roudaut et al. 2012). Circumferential endings are encircling all of three types

of hair follicles (Zigzag, Guard, Awl/auchene) (Zimmerman, Bai and Ginty 2014). Each ending composes a large receptive field of the hairy skin, innervated mainly by Aβ-fibers or low-threshold mechanoreceptors (LTMRs) and highly sensitive to gentle stroking of skin (Bai et al. 2015).

The mechanical sensitivity is naturally sense of mammals. The mechanism of mechanosensitivity still being studied for more understanding of physiological sensation. For the dental sciences, study of dental mechanical sensitivity is an important key to improve clinical treatment in tooth pathological condition.

3. Piezo mechanosensitive ion channels

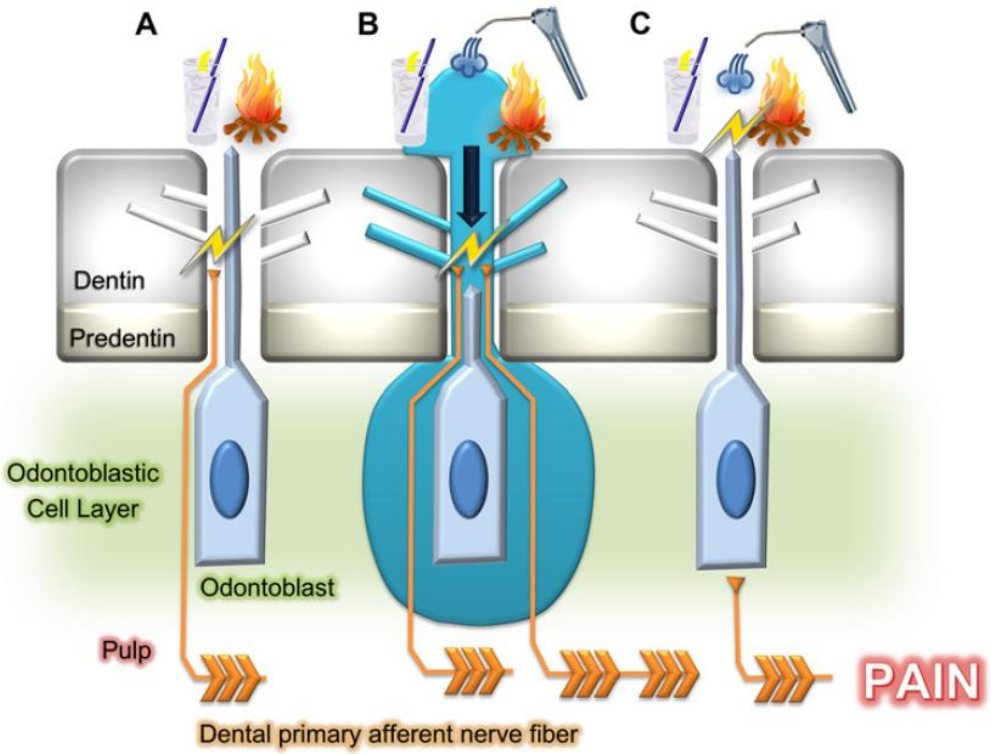
The mechanical sensations in vertebrate are transduced by many molecular mechanism in somatosensory neurons but have been rarely understood until Adam Patapoutin laboratory has screened the candidate mechanical ion channels by small interfering RNA mediated knockdown in mouse neuroblastoma cell line (N2A), and found a reduction in mechanically activated (MA) current. They named Piezo1 gene and next found its homologue Piezo2 channel (Coste et al. 2010). The Piezo mechanically activated (MA) current was recorded in mouse neuroblastoma cell line (N2A) and human embryonic kidney (HEK) cells and drosophila melanogaster (Coste et al. 2010; Kim et al. 2012). Piezos are large transmembrane proteins over 2.000 residues that consist 24-36 predicted transmembrane domains (Fig. 2), both

proteins highly expressed in lung. However, many studies have found Piezo1 in internal organs including endothelial innervate of blood vessel, uroepithelium, urinary bladder, kidney, heart and erythrocytes to serve as function in regulation of internal organs such blood flow, urine flow, heartbeat and volume-aging of erythrocytes (Bagriantsev, Gracheva and Gallagher 2014; Li et al. 2014; Miyamoto et al. 2014; Peyronnet et al. 2013; Ranade et al. 2014a). Whereas, Piezo2 or Fam38B was found in somatosensory neurons of mammals by in situ hybridization subpopulation of mouse DRG neurons (Coste et al. 2010), and corneal afferent neurons in trigeminal ganglion of guinea pig (Bron et al. 2014). Piezo2, a mechanically activated cation channel is also expressed in Merkel cells and nerve endings in the skin (Ikeda et al. 2014; Woo et al. 2014). The expression of Piezo2 serves a role of proprioception in mammals. Piezo2 knockout mouse showed a reduction in proprioceptive neurons in DRG and mesencephalic trigeminal nucleus (MTN) resulting in unconventional of limb positions, reduction in ability of balance and limbs hanging (Florez-Paz et al. 2016; Woo et al. 2015). In conclusion, Piezo2 has a potential role in somatosensory mechanotransduction as well as a low-threshold mechanoreceptors (LTMRS) (Fig. 3). The role of Piezo2 in pain was suggested in a previous study, which found Piezo2 mRNA co-expression with subpopulation of nociceptive neurons marker TRPV1 (Coste et al. 2010). Piezo2 was enhanced by modulator cAMP sensor Epac1 induce mechanical allodynia in neuropathic pain (Eijkelkamp et al. 2013). The mechanical stimuli transduction in endothelial cells by Piezo2 induced enhancement of hyperalgesia (Ferrari et al. 2015). In an inflammation condition, Piezo2 activation may praised by

inflammatory mediator (Bradykinin) in intracellular signals mediated mechanical hyperalgesia (Dubin et al. 2012).

4. Odontoblasts

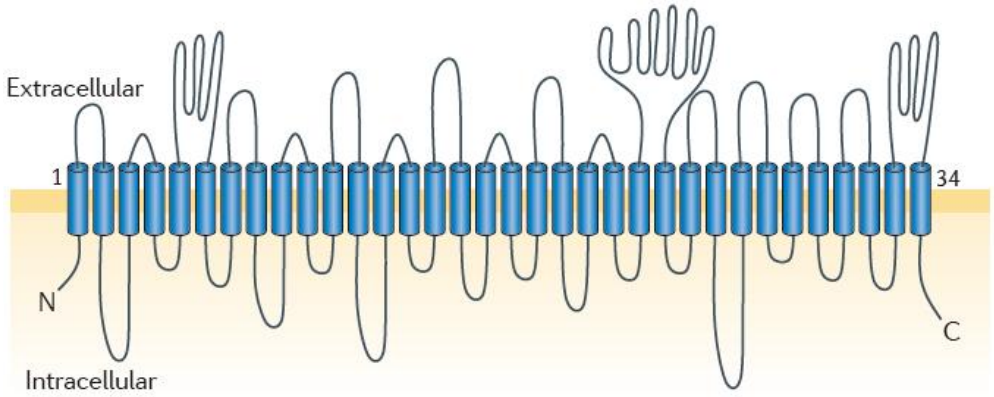
Odontoblasts are derived from cranial neural crest connective ectomesenchymal cells. These cells are responsible for mineralization in the primary dentinogenesis in early stage of tooth development, and secondary dentinogenesis after tooth eruption. Mature odontoblasts are the single layer of cells surrounding outer part of tooth pulp close to dentin, sending their processes to the dentinal tubules (Fig. 4). In generalizations, odontoblasts serve a critical role in dentinogenesis. Additionally, it may serve a role in immune response (Allard et al. 2006; Farges et al. 2009) and dental sensitivity. The functional expression of sensory ion channels as TRP channels (Chung, Jung and Oh 2013; Egbuniwe et al. 2014), sodium channels (Allard et al. 2006), potassium channels (Magloire et al. 2009), ASICs channels (Sole-Magdalena et al. 2011) and purinergic receptors have been found in odontoblasts (Lee et al. 2017; Nishiyama et al. 2016; Shibukawa et al. 2015).



J Dent Res reviews 92(11): 948-955, 2013

Fig. 1. Three hypotheses to explain dental nociception.

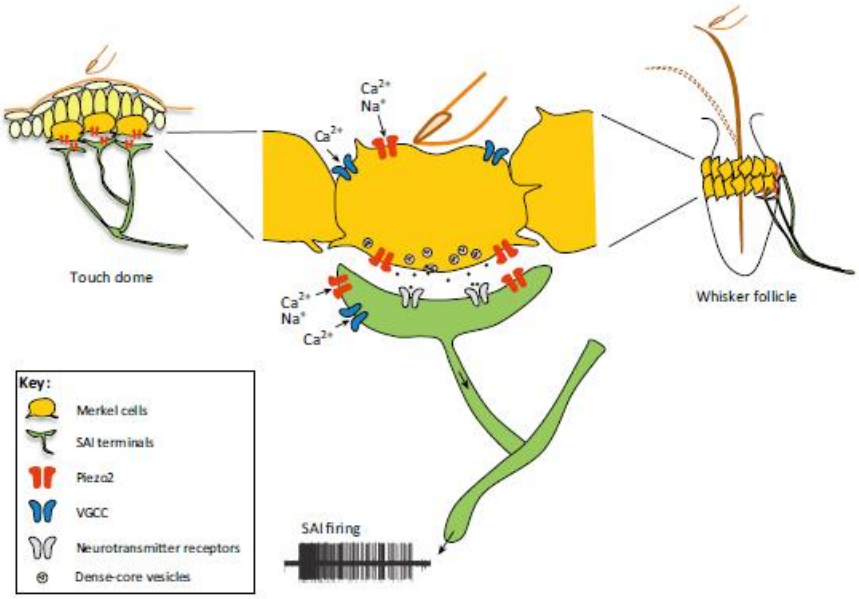
A) Neural theory: Nerve endings in the dentinal tubule are directly activated by external stimuli. **B)** Hydrodynamic theory: Fluid movement within dentinal tubules is detected by nerve endings. **C)** Odontoblast transducer theory: Odontoblasts act as pain receptors, reminiscent of the role of taste receptor cells in taste buds (Chung, Jung and Oh 2013).



Nature reviews Neuroscience 12(3): 139-153, 2011

Fig. 2. Piezo2 protein in mammalian.

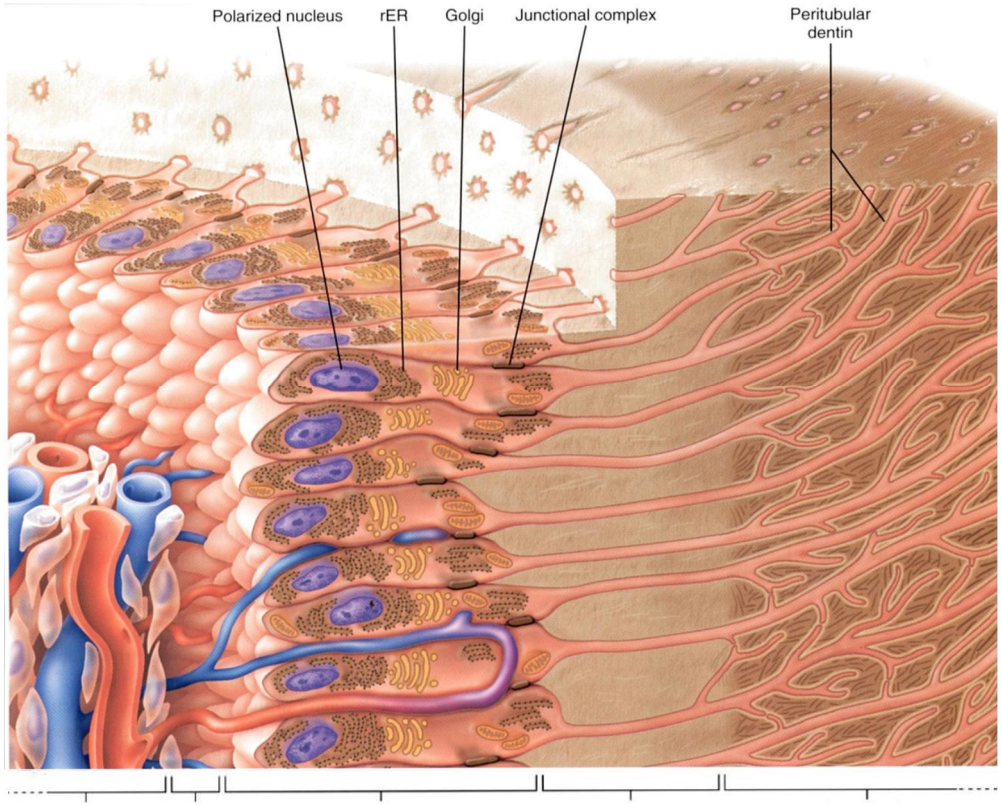
Hypothetical topology of mouse Piezo2. The plasma membrane expression of Piezo2 protein that consist 34 transmembrane domains, 3 large extracellular loops and cytoplasmic amino (N) and carboxyl (C) termini (Delmas, Hao and Rodat-Despoix 2011).



Trends in cell biology 25(2): 74-81, 2015

Fig. 3. A model of touch transduction in the Merkel cell neurite complex.

Gentle pressure on the skin or hair deformation of the whisker opens mechanotransduction channels, hypothesized to be Piezo2, in slowly adapting type I (SAI) afferents to initiate SAI action potential firing. **2)** Simultaneously, it opens Piezo2 channels in Merkel cells, which causes Merkel cell depolarization. **3)** Voltage-gated calcium channels (VGCC) in Merkel cells are subsequently activated, and **4)** neurotransmitters are released as a result and contribute to SAI firing (Woo, Lumpkin and Patapoutian 2015).



Oral histology 7(8):191-238, 2008

Fig. 4. Schematic representation the outer part of tooth pulp.

The illustration above represents cell rich zone, cell free zone, odontoblasts layer, predentin, and dentin. Odontoblast extend the processes into dentin through dentinal tubules (Nanci and Cate 2008).

Cell-rich Cell-free Odontoblast layer Predentin Mineralization

PURPOSE

Mechanical sensation originates from tooth pulp is initiated from the mechanoreceptors located in the nerve terminal of the pulp. These receptors transduce mechanical stimuli into neuronal signals which are transmitted to the brainstem via trigeminal ganglion neurons. Piezos ion channels are well described for their mechanosensitive properties, allowing mechanosensation in mammals. In dental sensory system, they may be involved in mechanosensitive pathway. My study will elucidate if Piezo mechanosensitive ion channels transduce mechanical stimuli in pulpal sensory system.

1. To investigate if Piezo ion channels are expressed in pulpal sensory system, including dental primary afferent neurons and odontoblasts.
2. To investigate if Piezo2 is expressed in dental primary afferent neurons, and compare their differential expression in nociceptors and mechanoreceptors.

INTRODUCTION

Dental pain is elicited by various stimuli, such as noxious heat/cold or light air puff. The functional and molecular studies on dental primary afferents neurons have identified ion channels such as TRP channels, Acid-sensing ion channels (ASICs) or purinoceptor channels (P2X3) which are activated by temperature, acids or inflammatory mediators (Chung, Jung and Oh 2013; Cook et al. 1997). Interestingly, not only noxious stimuli but also more subtle stimuli such as air puff or dentin probing onto teeth induce pain known as dental hypersensitivity. Dental hypersensitivity is classically explained by hydrodynamic theory, which hypothesizes the presence of mechanotransducers in the tooth pulp (Brännström and Åström 1964). However, the presence of mechanotransducers is yet to be identified in dental primary afferent (DPA) neurons.

Among the mechanotransducers identified to date, Piezo ion channels are the first to be identified in mammalian cells, particularly Piezo1 is expressed mostly in internal organs (Bagriantsev, Gracheva and Gallagher 2014; Miyamoto et al. 2014; Peyronnet et al. 2013; Ranade et al. 2014a), whereas Piezo2 is expressed mostly in dorsal root ganglion (DRG) neurons. Piezo2-expressing neurons display a rapidly inactivating (RI) current in response to mechanical stimuli (Coste et al. 2010; Ikeda et al. 2014). Recent studies have shown that Piezo2 is preferentially found in medium- to large-sized A-fibers and is crucial for light touch sensation (Bron et al. 2014; Ikeda et al. 2014; Ranade et al. 2014b). As dentinal tubules and the outermost

pulp are mostly innervated by pulpal A-fibers (Jyvasjarvi and Kniffki 1987), Piezo2 may be expressed in such DPA neurons to mediate mechanotransduction.

In this study, I evaluated the expression of Piezo mechanosensitive ion channels in dental primary afferent system and hypothesized that Piezo2 is expressed in dental primary afferent neurons. To prove this hypothesis, I selectively labeled rat dental primary afferent neurons innervating the upper molars with fluorescent tracer. The expression of Piezo ion channles was confirmed by reverse transcription polymerase chain reaction, in situ hybridization, single cell reverse transcription polymerase chain reaction and whole cell patch clamp. Finally, to suggest a possible mechanism in dental hypersensitivity which is associated with an intense level of pain elicited by an innocuous stimuli, I evaluated Piezo2 expression with TRPV1, a representative ion channel expressed in nociceptors, CGRP and IB4 the marker of nociceptive neurons, peripherin and neurofilament 200 (NF200) the cytoskeleton neurofilament a differentiation of neuronal size markers.

METERIALS AND METHODS

1. Animals

All surgical and experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Seoul National University. Male Sprague-Dawley rats (Orient Bio Inc., Sungnam, Korea) at 5 weeks of age (150-200 g) were used for the experiments. 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.

2. Odontoblasts isolation

Odontoblasts were prepared from adult (300-400 g) Sprague-Dawley rats (Orient Bio Inc., Sungnam, Korea) according to modified methods described previously (Guo et al. 2000; Yeon et al. 2009). Briefly, upper and lower incisors were extracted within 5-10 minutes of death and were kept in cold (3-5°C) extracellular solution (ECS) consisted of 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, and 10 mM HEPES, adjusted to pH 7.3-7.4 with NaOH. The standard enzyme solution consisted of collagenase IA (3 mg/mL) and protease I (0.25 mg/mL) in Ca^{2+}- and Mg^{2+}-free ECS. After removed the surrounding soft tissues, teeth were sectioned transversely with 500 μm thickness and pulled the pulp out

under microscope. Pulp tissue and tooth slices were incubated in 3 ml standard enzyme solution for 25 min at 37°C, raised two times with ECS and then triturated with a series of Pasteur pipettes. The triturated suspension was centrifuged at 1000 rpm for 5 minutes, and the supernatant were replaced on glass coverslips precoated 100 μg/ml poly-D-lysine following by cell and tissue adhesive 1μl Corning Cell-Tak (Discovery Labware, Inc., USA) with fresh extracellular solution and kept at 3-5°C until used.

3. Retrograde labeling

Dental primary afferent neurons were labeled as previously described (Kim et al. 2011). Briefly, the four upper first and second molar tooth were drilled by round bur of low speed (Malathon-3, USA) till the tooth pulp, and the DPA neurons were retrograde-labeled by applying a crystal of fluorescent dye, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes, Eugene, OR, USA) into the cavities followed by sealed with a temporary sealing material (GC Fuji II; GC Corporation, Tokyo, Japan) to prevent spread of tracer. Rats were house two weeks for the further trials.

4. Trigeminal ganglion neurons preparation

After two weeks of dental primary afferent neurons DiI labelling, rat TG neurons were prepared as described previously (Fang et al. 2007). Briefly,

trigeminal ganglia were removed and washed several times in cold (4°C) Hanks' balanced salt solution (Invitrogen, Carlsbad, CA, USA) and then incubated for 30 min at 37°C in Hanks' balanced salt solution (Invitrogen, Carlsbad, CA, USA) containing collagenase (5 mg/ml)/dispase II (1 mg/ml) (Roche, Germany) for 30 minutes and subsequent 0.16% trypsin (Gibco, UK) for 7 minutes. The cells were washed in Dulbecco's modified Eagle's medium (Gibco, UK) and triturated with a flame-polished Pasteur pipette to separate cells and remove processes. Subsequently, cells were centrifuged, resuspended and placed on glass coverslips (12 mm in diameter) coated by poly-L-ornithine (0.5 mg/ml; Sigma Aldrich, ST. Louis, MO, USA) and the cells were maintained in an incubator at 37°C equilibrated with 5% CO_2 until use.

5. Reverse transcription–polymerase chain reaction

To assess Piezo1 and Piezo2 expression in rat whole TG and tooth pulp, total RNA was isolated using the RNeasy mini kit (Qiagen, Germany). For positive control, lung mRNA was isolated and negative control white blood cells (WBC) from 10 ml blood was collected. The collected blood was centrifuged at 3000 rpm for 10 minutes (Labogene 1248) to separate white blood cells layer from red blood cells (RBC) and plasma. The separated WBC layer was slowly aspirated transfer to 1.5 ml eppendorf tube for mRNA isolation. Complementary DNA was synthesized from 1 μg of total RNA by M-MLV reverse Transcriptase (Invitrogen) according to manufacturer's protocols. The following PCR primers were used (Table 1. Outer

primers). PCR amplification was done by Accupower^{TM} Hotstart PCR Premix (Bioneer Corp., Deajeon, Korea). The PCR products were then displayed on the Safe-Pinky (GenDEPOT, USA) stained 1.5% agarose gel and photographed using a UV digital camera.

6. Single-cell reverse transcription-polymerase chain reaction (ScRT-PCR)

Single-cell reverse transcription-polymerase chain reaction (ScRT-PCR) was performed as previously described (Park et al. 2006). Briefly, the targeted cells were collected by a patch pipette with tip diameter of about 10-20 μm and put into reaction tube containing reverse transcription agents. Reverse transcription was incubated 10 minutes at 25˚C, 1 hour and 30 minutes at 50˚C, then 85˚C 5 minutes and removed mRNA during cDNA synthesis by using RNase H 37˚C 20 minutes (T100 Thermal Cycler, BIO-RAD). The cDNA products were stored at -20˚C until PCRs processing. All PCR amplifications were performed with nested primers (Table 1). The first round of PCR was performed in 50 μl of PCR buffer containing 0.2 mM dNTPs (Invitrogen, Carlsbad, CA, USA), 0.2 μM ‘‘outer’’ primers, 1~3 μl RT product, and 0.2 μl platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA). For the second round of amplification, the reaction buffer (50 μl) contained 0.2 mM dNTPs, 0.2 μM ‘‘inner’’ primers, 2 μl products from the first round, and 0.2 μl platinum Taq DNA polymerase. The PCR products were then displayed on the

ethidium bromide-stained 1.5% agarose gel. Photographed using a UV digital camera.

7. Western blot

Adult SD rat (6-8 weeks old, male) was deeply anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and perfused transcardially with 0.1 M PBS with heparin 500 U/L. Four incisor were extracted and cut horizontally into 3mm pieces. The pulp tissue were pulled out, and further trigeminal ganglion and lung tissue were manipulated on ice for further preparation except when stated otherwise. The tissues were homogenized in protein extraction buffer containing 10 mM Tris-HCl, 5 mM EDTA, 300 mM sucrose, protease inhibitor cocktail (PIC) (Sigma-Aldrich), pH 7.5. The homogenates were centrifuged at 600 g for 10 min at 4°C and then supernatant was collected. After the re-centrifugation of the above supernatant at 23000 g for 30 min at 4°C, supernatant was discarded and pellet was resuspended in protein extraction buffer to obtain enriched membrane protein. Protein concentration was determined with Lowry assay reagent (Biorad, USA) and quantified with a plate reader. Each samples of 40ug were mixed with 6x SDS sample buffer followed by boiling for 5 min at 95°C. For gel electrophoresis, the proteins were separated on 5% stacking/8% resolving gels by SDS-PAGE and then transferred onto Polyvinylidene difluoride (PVDF) membrane (Biorad). The membrane was blocked with tris-buffered saline-Tween 20 (TBS-T) based protein free blocking buffer(Thermo) for Piezo1, Piezo2 and with 5% skim milk in tris

buffer saline containing 0.1% Tween20(TBS-T), pH 7.4 for β-actin at room temperature for 1 hour and subsequently incubated with goat anti-Piezo1(Santa Cruz, 1:100), rabbit anti-Piezo2(Santa Cruz, 1:100), mouse anti-β-actin(Sigma, 1:10000) at 4°C overnight. The membrane was washed with TBS-T and then incubated with anti-rabbit IgG-HRP, anti-mouse IgG-HRP respectively for 1hr at room temperature. After incubation, the membrane was washed with TBS-T then exposed to UV after chemiluminescence reagent (Amersham) treatment. Images were attained with Imagelab.

8. In situ hybridization

Mouse DPA neurons were retrograde-labeled by filling fluorescent tracer FluoroGold (FG, 2 μl of 4% solution in distilled water, Biotium) into an upper molar under ketamine anesthesia (80 mg/kg, i.p.). Two weeks later, the mice were perfusion fixed with 4% paraformaldehyde in 0.1% DEPC-treated PBS (pH 7.4). The harvested TG was post-fixed for 2 hours in the same fixative, then cyroprotected with 30% sucrose in DEPC-treated PBS for 48 hours at 4°C. Serial frozen transverse sections (14 μm thickness) were mounted on slide glasses for further processing. For fluorescent in situ hybridization, I used the Mm-Piezo2 probe (Advanced Cell Diagnostics, Cat. 400191), and RNAscope® Multiplex Fluorescent Reagents Kit (Cat. 320850) according to the manufacturer's instructions (ACD Technical Notes #320511 for tissue preparation, and #320293 for

fluorescent labeling). The mounted sections were examined by fluorescence microscopy.

9. Whole Cell Patch Clamp

Whole cell recordings were made with Axopatch 200B amplifier and Digidata 1322A (Molecular Devices), filtered at 1-2 kHz and sampled at 10 kHz. The pipette solution for voltage clamp recordings was consisted of the following (in mM): 125 CsCl, 1 $MgCl_2$, 4.8 $CaCl_2$, 0.4 NaGTP, 4 MgATP, 1 $MgCl_2$, 10 HEPES, and 10 EGTA, adjusted to pH 7.3 with CsOH (Hao and Delmas 2010). The external solution consisted of the following (in mM): 132 NaCl, 3 KCl, 1 $MgCl_2$, 2.5 $CaCl_2$, 10 HEPES and 10 D-glucose, adjusted to pH 7.3 with NaOH (Hao and Delmas 2010). The resistance of the pipettes was 2.5~3.5MΩ. Inward currents were recorded at a holding potential of -60 mV. For IB4 staining, the primary cultured TG neurons were incubated in 37°C DMEM with fluorescent isothiocyanate conjugated IB4 (IB4-FITC, Sigma Aldrich, L2895, 5 μg/ml) for 30 min. Capsaicin was prepared in ethanol as stock solution of 10 mM and was diluted for voltage clamp analysis to the final concentration of 500 nM. The extracellular solution was gravity fed by a rate of 1.5-2 ml/min. All the chemicals for preparing the above solutions were purchased from Sigma Aldrich unless stated otherwise.

10. Mechanical stimulation and Analysis

Patch pipettes pulled for whole cell patch clamp were fire-polished to make a closed tip with a 3 to 4 μm diameter. The pipette was attached to an amplifier headstage (Molecular Devices), which was mounted on a micromanipulator that can be computer operated in 0.4 to 1 μm steps in horizontal direction. The mechanical stimulation was delivered either sporadically (Scientifica) or in a time locked manner (Piezosystem Jena), with the speed of 625 μm/sec (Hao and Delmas 2010). The forward movement was maintained for 0.2~1s until the probe was removed back to its original position. The mechanical stress was relieved for more than ten seconds until the successive trial. The inactivation kinetics were fitted to exponentials using the Chebyschev nonlinear least square fitting procedure provided in pClamp 10 software.

11. Piezo2 knockdown and quantitative PCR

Primary cultured TG neurons were transfected with 10 nM Piezo2 (Silencer Select, Ambion, 4390771) or scrambled (Silencer Select, Ambion, 4390843) siRNA 1 hr after plating. Transfection was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. 36 hours post transfection, the cells were used for whole cell recording or RNA extraction to evaluate Piezo2 knockdown. For whole cell recording, the experimenter was blinded to the siRNA treatment.

Total RNA was extracted by Trizol (Invitrogen) and cDNA was synthesized from total RNA by M-MLV reverse Transcriptase (Invitrogen), according to manufacturer's protocols. For qPCR, 10 ng of cDNA was used. Primers used are listed in Table 1. Each sample (20 μl) was run in triplicates, using SYBR Master Mix (Invitrogen) according to the manufacturer's instructions. PCR was performed on 7500 Real-Time PCR system (Applied Biosystems) under conditions as : 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 sec, 55°C for 15 sec, and 72°C for 1 min. Piezo2 expression difference was analyzed with ΔΔCT values with GAPDH as reference gene.

12.Statistical Analysis

All of the data are presented as mean ± SEM unless stated otherwise. Statistical analysis were performed with Prism software except the fitting procedure for inactivation kinetics analysis. Statistical significance of difference in cell size was analyzed by two-tailed unpaired t-test. The criteria for significance was $p < 0.005$.

Table 1. The list of primers used

ScRT-PCR

Target gene	Outer primers	Inner primers	GenBank No.
GAPDH (316 bp)	AGGTCATCCCAGAGCTAACG CACCCTGTTGCTGTAGCCATAT		NM_017008
DSPP (452, 408 bp)	GCTGAGGTGACACCAAGCATT ACTTTTGTTGCCCGTGCTG	GGAAGGTGCTGGTTTGGATAAT ACCTTCGGTTTCTAATCCCTGA	NM_012790
Piezo1 (462, 255 bp)	TACCTTGGTGTGCGTATCCA GCTGGGCTCCTACTCCTTCT	TTGTGGGGCTCTACGTCTCT TGGGCTCCTACTCCTTCTCA	NM_001077200
Piezo2 (229, 116 bp)	AGTAGCGGAACTGGAAGGGA GCCAGCAATCATTTTGGCTA	ACCGACCCCAATAGTTGCTT GCATCCGTGAGAGGAAAAGA	XM_006222595
TRPV1 (462, 245 bp)	ATGCCAGCTACACAGACAGCTA CCTTCCTGTTGGTGATCTCTTC	TGACCCTCTTGGTGGAGAAT TGTGTTATCTGCCACCTCCA	NM_031982
$Na_v1.8$ (356, 215 bp)	AGATCCACTGTCTGGACATCCT GAAAGACGTAGCAGAGGCAGTT	CCAATCTCTCCAAAGCATCC GAGTCCACTGTTTGCCATGA	NM_017247
PRPH (311, 255 bp)	CCTCTCCCTGATGCATTGAT AGCCTGGGGTACACATGAAG	GAAGAAACCCAGGACAGCAG AGCCTGGGGTACACATGAAG	M62632
NF200 (348, 269 bp)	CGAGAGGGACACCCAGAATA TCGGTAGGACATACGGGAAG	GGACACCCAGAATAGCCAAA GTGGAGCGTTCAGCAATACA	NM_012607
CGRP	ACCTTCGGGTCTGAGGAACT	TCGGGTCTGAGGAACTAACG CCAGCAGGAGACACCATCAT	NM_138513

(334, 261 bp)	GGTTCACCAGAGCTGCTACC		

qPCR

Target gene	Outer primers	Inner primers	GenBank No.
GAPDH (118 bp)	CAACTCCCTCAAGATTGTCAGCAA GGCATGGACTGTGGTCATGA		NM_017008
Piezo2 (116 bp)	ACCGACCCCAATAGTTGCTT GCATCCGTGAGAGGAAAAGA		XM_006222595

Table 2. Classification of recorded DPA neurons based on decay time constant of mechanosensitive currents.

Decay constent (ms)	Tau (mean ± SEM)	Recorded neurons	Percentage	Cell size (mean ± SEM)	Abriviation
$\tau < 10$	4.6 ± 0.3	90	40.0	29.0 ± 0.5	RI
$10 < \tau < 30$	18.0 ± 1.2	26	11.6	31.4 ± 0.8	nRI
$30 < \tau < 100$	49.4 ± 5.0	10	4.4	26.3 ± 1.2	
$\tau > 100$	274.1 ± 90.4	5	2.2	30.1 ± 2.3	
Non-responsive	-	94	41.8	25.2 ± 0.5	NR
Total	-	225	100	-	-

RI: rapidly inactivating

nRI: non-rapidly inactivating

NR: non-responsive

RESULTS

1. Piezos molecular expression in odontoblast

I first evaluated Piezo proteins and mRNA expression in tooth pulp and odontoblasts. Dental pulp was analyzed by Western blot showing expression of Piezo1 and Piezo2 (Fig. 5A), as well mRNA expression by RT-PCR (Fig. 5B). Whereas lack of both Piezo1 (n = 0/24) and Piezo2 (n = 1/24) mRNA expression were confirmed by single cell RT-PCR of odontoblasts (Fig. 5C).

2. Piezos molecular expression in DPA neurons

I again confirmed the expression of Piezo1 and Piezo2 proteins and mRNA by using Western blot analysis in trigeminal ganglion, the data not showed Piezo1 protein, only Piezo2 protein expression in trigeminal ganglion (TG). Lung was a positive proteins expression of Piezo1 and Piezo2. Predicted protein sizes was 233 kDa for Piezo1, 300 kDa for Piezo2 and 43 kDa for β-actin (Fig. 6A). I further investigated mRNA expression by RT-PCR was showed both of Piezo1 (255 bp)

and Piezo2 (229 bp) mRNA in trigeminal ganglion (Fig. 6B). Single-cell RT-PCR shows Piezo1 mRNA expression in 10% of DPA neurons (n = 3/30) and majority were Piezo2 mRNA expression (Fig. 6C, 8B). Predict primer length Piezo1 (255 bp), Piezo2 (116 bp). Lung is positive control.

3. Molecular expression of Piezo2 in dental primary afferent neurons

I confirmed the expression of Piezo2 mRNA in rat TG by RT-PCR analysis. Lung and white blood cells were used as positive and negative control, respectively, according to the mRNA expression database for Piezo2 (Coste et al. 2010; www.genecards.org; Fig. 6B). Piezo2 mRNA in mouse DPA neurons was then visualized by fluorescent in situ hybridization analysis in the maxillary division of retrograde-labeled ipsilateral TG, which appeared as red puncta in the cytosolic compartment of the neurons (Fig. 7A). Piezo2 mRNA fluorescence was observed in 56% of FG labeled DPA neurons (n = 99/176) with Piezo2 mRNA-positive DPA neurons significantly larger on average than Piezo2 mRNA-negative neurons (Fig. 7B, 7C; 37.0 ± 1.0 μm and 29.2 ± 1.1 μm in diameter, respectively; Student's t test, $p < 0.0001$).

4. Molecular characterization of Piezo2 mRNA-positive dental primary afferents neurons

I determined whether DPA neurons express Piezo2 by single-cell RT-PCR (Fig. 8B). Piezo2 mRNA was detected in 71% (n = 120/170) of DPA neurons, with these neurons mostly being medium-to large sized (Fig. 8C). Piezo2 positive DPA neurons were significantly larger than Piezo2 negative DPA neurons (Piezo2 positive vs. negative; 31.61 ± 0.70 μm vs. 24.69 ± 0.94 μm in diameter ($p<0.0001$, data not shown), with higher Piezo2 expression in medium- to large-sized DPA neurons (Fig. 8D). Piezo2 positive DPA neurons were further analyzed for mRNA expression of various nociceptive markers. As sensory neurons can be classified as nociceptors by their expression of receptors for noxious stimuli, conduction properties, level of myelination, or neurotransmitter contents, I have focused on the co-expression of transient receptor potential vanilloid 1 (TRPV1), voltage gated sodium channel 1.8 (Nav1.8), calcitonin-gene related peptide (CGRP), peripherin and neurofilament 200 kD (NF200) in Piezo2-positive population (Chiu et al. 2014; Fried, Sessle and Devor 2011; Ho and O'Leary 2011; Kim et al. 2011; McCarthy and Lawson 1990; Vang et al. 2012) (Fig. 8B). DPA neurons were also examined for IB4 positivity before collection for later analysis (Stucky and Lewin 1999). The mRNA expression of TRPV1, Nav1.8, CGRP, peripherin, NF200 were found in 44% (n = 62/141), 69% (n = 49/71), 87% (n = 31/27), 25% (n = 15/61), 86% (n = 61/71) of DPA neurons respectively, and IB4 positivity was observed in 18% (n = 17/92) of DPA neurons (data not shown). Among Piezo2 positive DPA neurons, the

mRNA for TRPV1, Nav1.8, CGRP, peripherin, NF200 were observed in 48% (n = 45/94), 89% (n = 42/47), 90% (n = 19/21), 29% (n = 12/41), 100% (n = 47/47) respectively. IB4 was positive in 14% of Piezo2 positive DPA neurons (n = 3/21) (Fig. 8E).

5. Mechanosensitive Piezo2-like currents are observed from dental primary afferent neurons

I next measured mechanosensitive currents in DPA neurons by whole-cell patch-clamp recordings (Fig. 9A). Mechanical stimulation evoked mechanosensitive currents in 58% of DPA neurons (n = 131/225, Fig. 9C). When mechanosensitive DPA neurons were classified based on the decaying kinetics of their mechanosensitive currents, Piezo2-like, rapidly inactivating (RI) currents with a decay time constant of <10 ms (τ < 10 ms) were observed in 40% (n = 90/225) of DPA neurons tested, occupying 69% (n = 90/131) of the mechanosensitive population (Fig. 9B, C; further information of recorded DPA neurons in Appendix Table 2). The average decay time constant of RI currents was 4.6 ±0.3 ms, consistent with previous studies (Fig. 9D) (Coste et al. 2010; Ranade et al. 2014b). The maximal amplitude of RI current obtained from individual DPA neurons increased according to cell size, whereas maximal RI currents smaller than <400 pA were also observed regardless of cell size (Fig. 9E). scRT-PCR analysis revealed

that 100% (n = 10/10) of DPA neurons with mechanosensitive RI currents had Piezo2 mRNA. Among non-mechanically responsive DPA neurons, 38% (n = 3/8) had Piezo2 mRNA (Fig. 9F).

6. Mechanosensitive, rapidly inactivating current in DPA neurons is mediated by Piezo2 ion channels

To confirm whether mechanosensitive RI currents in DPA neurons are generated by Piezo2 activation, I employed two approaches; pharmacological blocking of Piezo2 currents with 30 μM ruthenium red and siRNA mediated Piezo2 knockdown. Consistent with previous studies (Drew, Wood and Cesare 2002; Ikeda and Gu 2014), I found that RI currents were reversibly blocked by 30 μM ruthenium red ($p < 0.0001$, Fig. 10A and 10B). I also recorded RI currents in DPA neurons after transfection with either Piezo2 or scrambled siRNA. When Piezo2 mRNA level was significantly reduced by Piezo2 siRNA transfection ($n = 3$, $p < 0.0015$, compared to scrambled siRNA transfection, Fig. 10C), RI currents were only observed in 9% of the recorded Piezo2 siRNA-transfected neurons (n = 2/22) whereas 42% (n = 10/24) of the scrambled siRNA-transfected DPA neurons showed RI currents (Fig. 10D). When compared to scrambled siRNA-transfected DPA neurons, the amplitude of the RI currents were reduced after Piezo2 siRNA

transfection, although statistically insignificant (-721.0 ± 263.1 pA vs. -54.5 ± 16.5 pA, respectively, p = 0.3013, Fig. 10E, 10F).

7. Mechanosensitive, rapidly inactivating currents are robust in capsaicin-insensitive and IB4-negative dental primary afferent neurons

DPA neurons showing mechanosensitive RI currents were tested for capsaicin sensitivity and IB4 positivity. Among RI population, 55% (n = 16/29) were capsaicin-insensitive. RI currents were significantly larger in capsaicin-insensitive DPA neurons than capsaicin-sensitive counterparts (Fig. 11A -380.1 ± 82.5 vs. -118.3 ± 20.1 pA, respectively, p = 0.0094,). The cell size was larger in capsaicin-insensitive population than that of capsaicin-sensitive (29.9 ± 1.2 vs. 25.8 ± 1.2 μm, respectively, p = 0.0209, Fig. 11B). The decay constant of the RI currents in capsaicin-insensitive DPA neurons was slightly smaller than the sensitive neurons, but not significantly different (3.3 ± 0.7 -vs. 4.6 ± 0.9 ms, respectively, p = 0.2240, figure not shown). When DPA neurons were examined according to IB4 binding, mechanosensitive RI currents were observed only in IB4-negative population (100%, n = 22/22), as IB4-positive DPA neurons failed to show any mechanosensitive RI currents (28.5 ± 1.0 -vs. 25.8 ± 1.2 μm, respectively, p = 0.095, Fig. 11C).

FIGURES

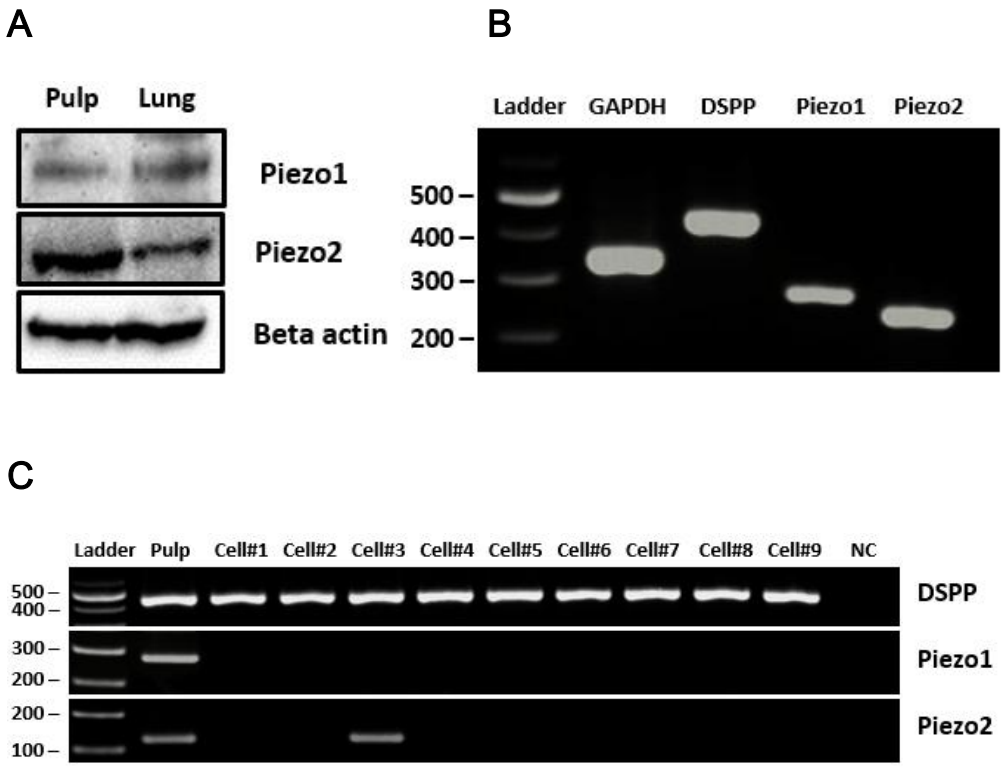
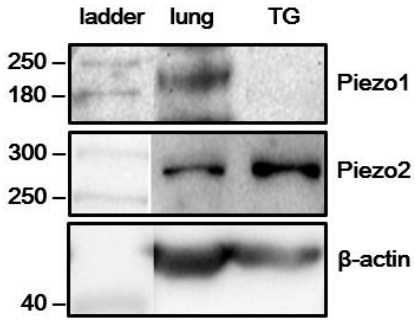


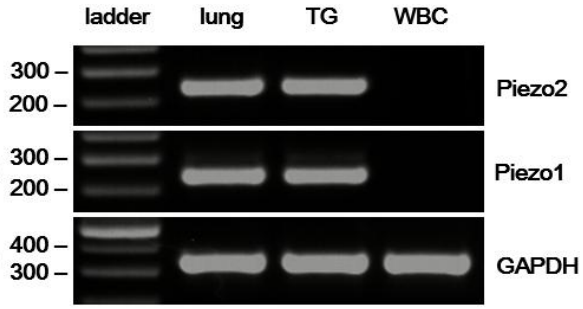
Fig. 5. Piezos molecular expression in odontoblasts

A) Western blot showed Piezo1 and Piezo2 proteins in dental pulp tissue and lung, a positive control. Predicted protein sizes were 233 kDa for Piezo1, 300 kDa for Piezo2 and 43 kDa for β-actin. **B)** RT-PCR showed mRNA expression in tooth pulp Piezo1 (255 bp) and Piezo2 (229 bp). **C)** Single cell RT-PCR data showed that lack of Piezo1 mRNA in odontoblasts, whereas 4% (n = 1/24) of cells expressed Piezo2 mRNA. Predict primer length DSPP (408 bp), Piezo1 (255 bp), Piezo2 (116 bp). Pulp is positive control.

A


ladder
lung
TG
250
180
Piezo1
300
250
Piezo2
β-actin
40

B


ladder
lung
TG
WBC
300
200
Piezo2
300
200
Piezo1
400
300
GAPDH

C

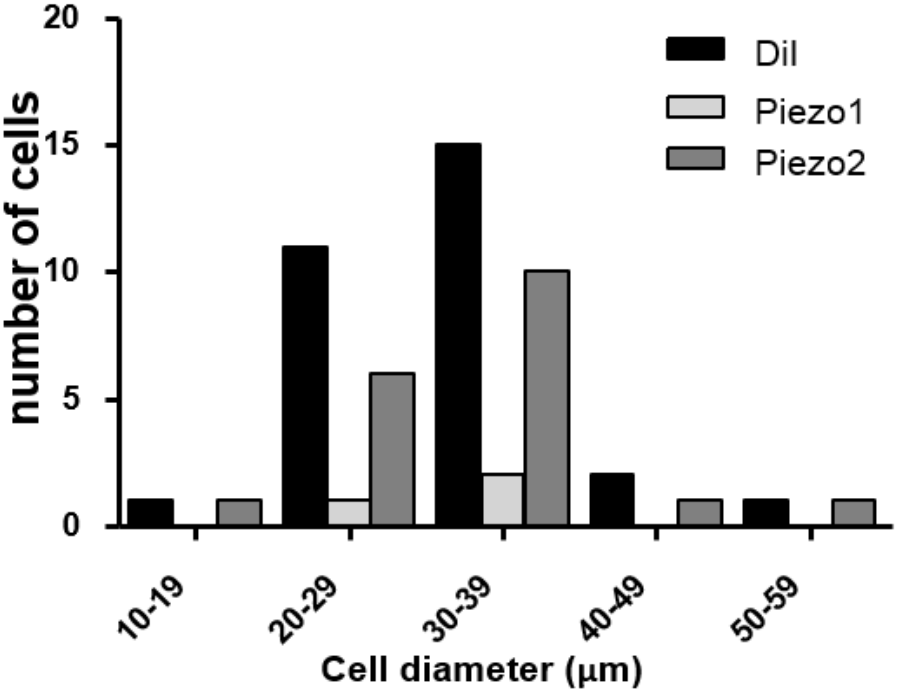
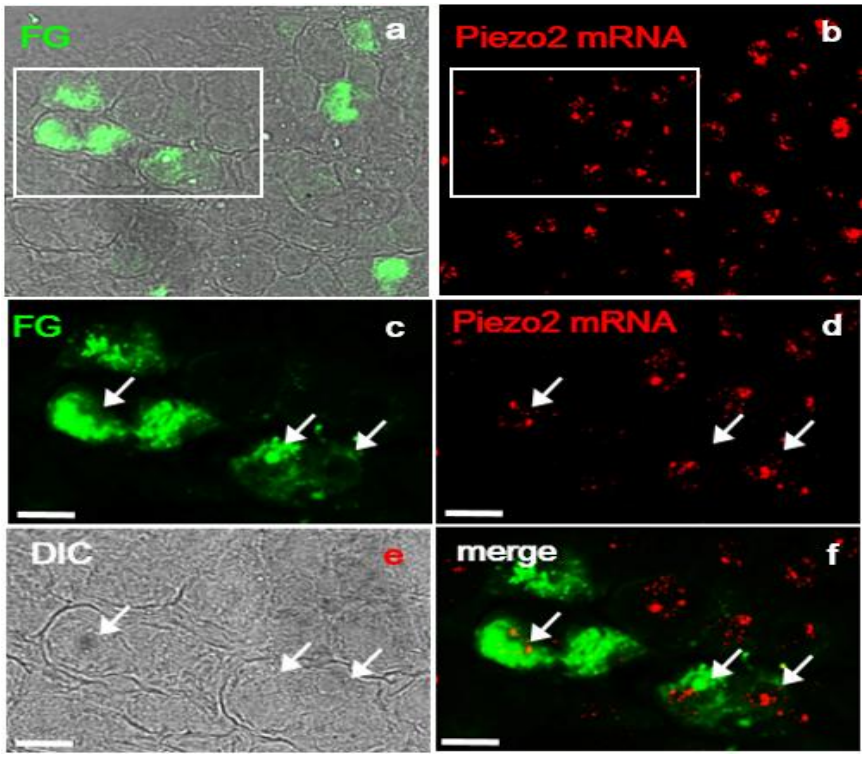

20
15
10
5
0
number of cells
DiI
Piezo1
Piezo2
10-19
20-29
30-39
40-49
50-59
Cell diameter (μm)

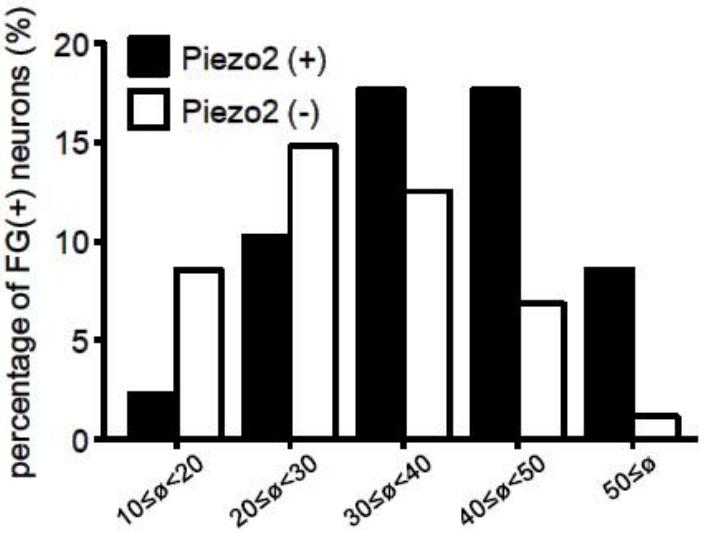
Fig. 6. Piezos molecular expression in DPA neurons

A) Western blot detected Piezo1 and Piezo2 proteins in TG. Lung was a positive control. Predicted protein sizes were 233 kDa for Piezo1 and 300 kDa for Piezo2 and 43 kDa for β-actin. **B)** RT-PCR analysis to detect Piezo1 and Piezo2 mRNA in TG. Lung and separated white blood cells (WBC) were positive and negative control for Piezo1 and Piezo2 expression. Predicted sizes were 255 bp for Piezo1, 229 bp for Piezo2, and 316 bp for GAPDH. **C)** Normalized of Piezo1 and Piezo2 mRNA expression in DiI-labeled dental primary afferent neurons (DPA).

A


FG
a
Piezo2 mRNA
b
FG
c
Piezo2 mRNA
d
DIC
e
merge
f

B


percentage of FG(+) neurons (%)
20
15
10
5
0
Piezo2 (+)
Piezo2 (-)
10≤ø<20
20≤ø<30
30≤ø<40
40≤ø<50
50≤ø

C

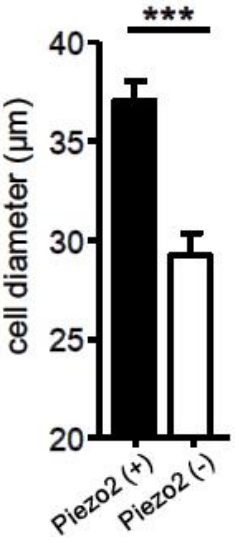

***
cell diameter (μm)
40
35
30
25
20
Piezo2 (+)
Piezo2 (-)

Fig. 7. Molecular expression of Piezo2 in DPA neurons

A) Photographs of FG-labeled DPA neurons (green) in whole TG section and Piezo2 mRNA as red fluorescent puncta. Aa) and Ab) are the same field from maxillary division of the TG with FG merged with DIC and Piezo2 mRNA, respectively. White squares in Aa) and Ab) are enlarged in panel Ac-f). Ac-f) Neurons in whole TG section showing FG (green), Piezo2 (red), merged and DIC image. Arrows highlight DPA neurons positive for Piezo2 mRNA. Scale bar: 25 μm. **B)** Size distribution of Piezo2 mRNA-positive and -negative DPA neurons. **C)** Diameter of DPA neurons according to Piezo2 mRNA positivity (Student's t test, $p < 0.001$).

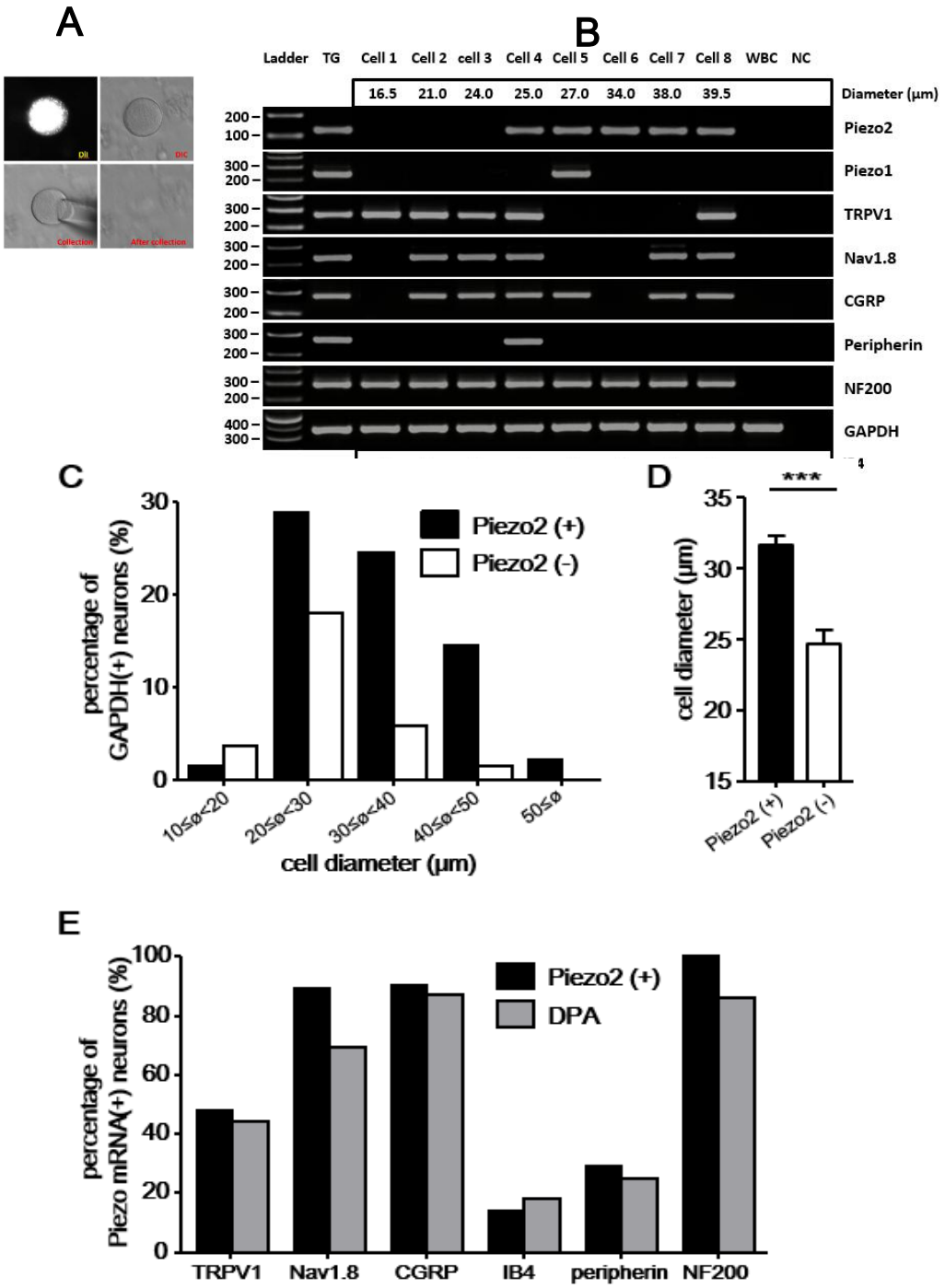

A
B
Ladder
TG
Cell 1
Cell 2
cell 3
Cell 4
Cell 5
Cell 6
Cell 7
Cell 8
WBC
NC
16.5
21.0
24.0
25.0
27.0
34.0
38.0
39.5
Diameter (μm)
Piezo2
Piezo1
TRPV1
Nav1.8
CGRP
Peripherin
NF200
GAPDH
DII
DIC
Collection
After collection
C
percentage of GAPDH(+) neurons (%)
Piezo2 (+)
Piezo2 (-)
10≤ø<20
20≤ø<30
30≤ø<40
40≤ø<50
50≤ø
cell diameter (μm)
D
***
cell diameter (μm)
Piezo2 (+)
Piezo2 (-)
E
percentage of Piezo mRNA(+) neurons (%)
Piezo2 (+)
DPA
TRPV1
Nav1.8
CGRP
IB4
peripherin
NF200

Fig. 8. Characterization of Piezo2 mRNA positive DiI-labeled DPA neurons by single-cell RT PCR.

A) DiI-labeled dental primary afferent neurons collection. **B)** The representative gels showed scRT-PCR products from eight individual DPA neurons. TG and single white blood cell (WBC) extracts were used as positive and negative controls, respectively. Predicted sizes for selected markers are Piezo1 (255 bp), Piezo2 (116 bp), TRPV1 (245 bp), Nav1.8 (215 bp), CGRP (261 bp), Peripherin (255 bp), NF200 (269 bp), GAPDH (316 bp). **C)** Size distribution of Piezo2 mRNA-positive and –negative DPA neurons. **D)** Diameter of DPA neurons according to Piezo2 mRNA positivity (Student's t test, $p < 0.001$). **E)** Summary of scRT-PCR results from Piezo2 mRNA-positive and total DPA neurons.

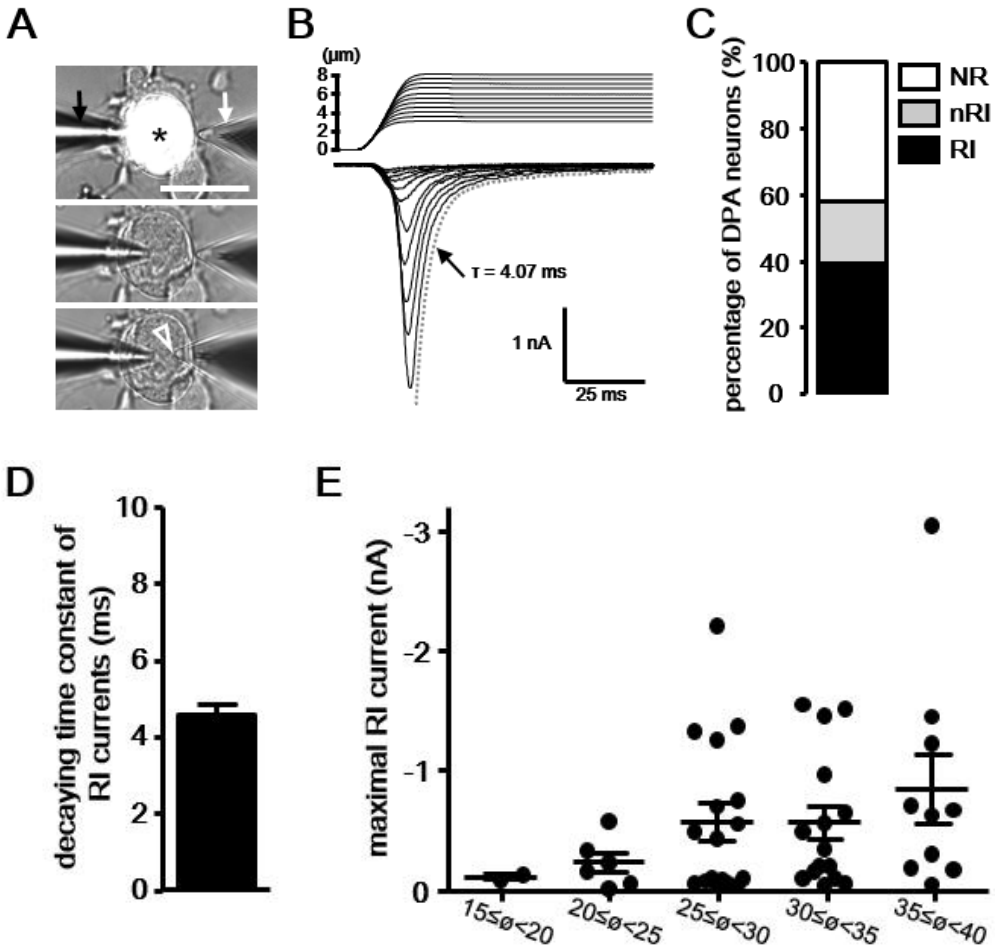

A
B
(μm)
8
6
4
2
0
τ = 4.07 ms
1 nA
25 ms
C
percentage of DPA neurons (%)
100
80
60
40
20
0
NR
nRI
RI
D
decaying time constant of RI currents (ms)
10
8
6
4
2
0
E
maximal RI current (nA)
-3
-2
-1
0
15≤ø<20
20≤ø<25
25≤ø<30
30≤ø<35
35≤ø<40
F
RI
RI
NR
RI
NR
ladder
1
2
3
4
5
NC
200
100
400
300
Piezo2
GAPDH

Fig. 9. Measurement of Piezo2-like mechanosensitive currents in DPA neurons.

A) Schematic for mechanical stimulation. Asterisk: DiI-labeled DPA neuron; Black arrow: patch pipette for whole cell patch clamp recording; White arrow: fire-polished glass pipette for mechanical stimulation; Open arrowhead: advanced pipette tip for mechanical stimulation. **B)** RI currents evoked by a series of 0.5 μm incremental steps towards the cell being recorded, with a decay time constant less than 10 ms. **C)** Classification of DPA neurons based on the decay time constant of mechanically evoked inward current. **D)** Average decay time constant of RI currents in DPA neurons. **E)** Average maximal amplitude of RI current according to cell size (One-way ANOVA followed by Bonferroni's post test, $p > 0.05$). **F)** Representative gels showing scRT-PCR products from recorded DPA neurons. Predicted sizes are 116 bp for Piezo2 and 316 pb for GAPDH. (RI: rapidly inactivating cells; nRI: non-rapidly inactivating cells; NR: non-responsive cells).

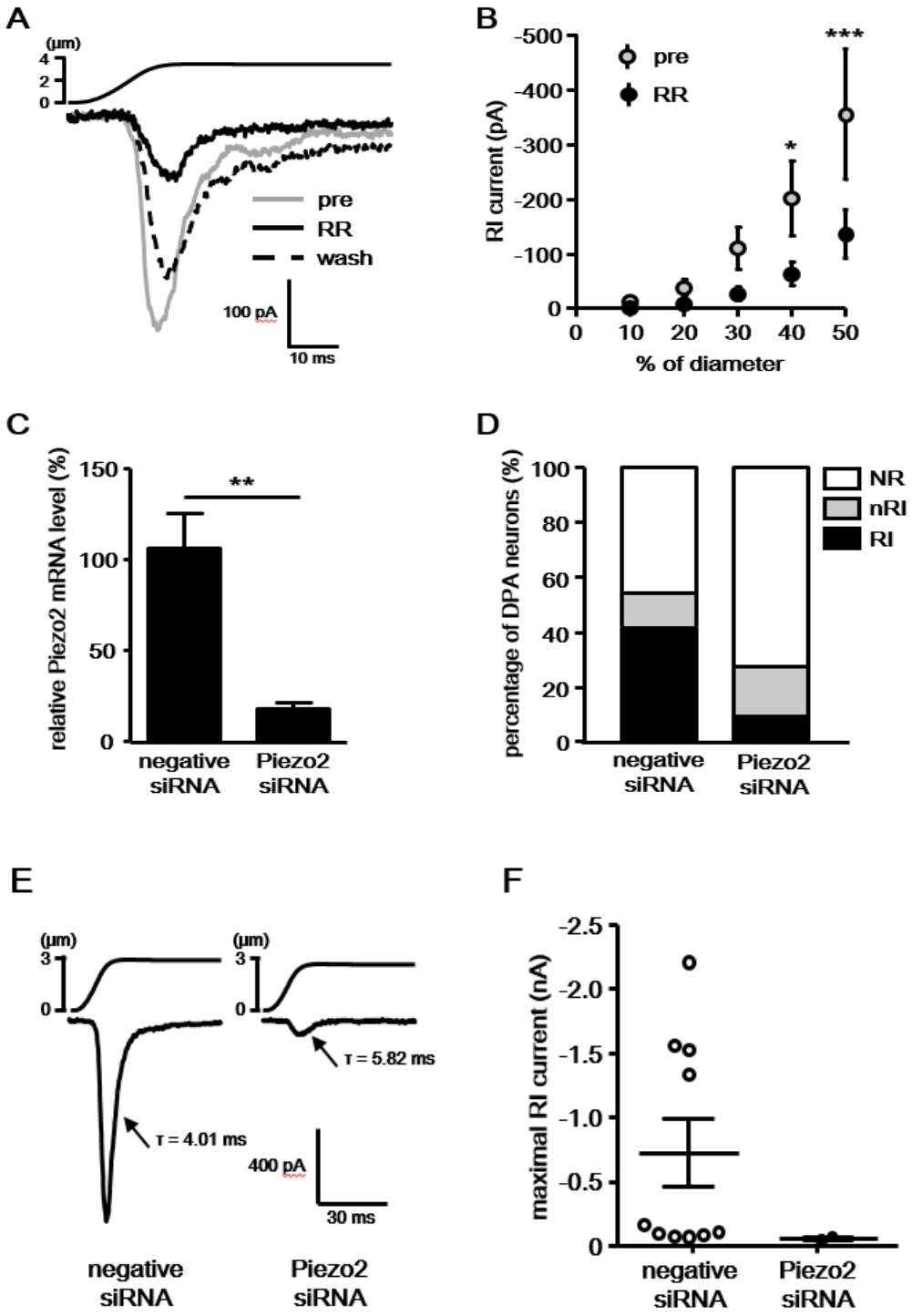

A
(μm)
4
2
0
pre
RR
wash
100 pA
10 ms
B
-500
-400
-300
-200
-100
0
RI current (pA)
pre
RR
*
***
0
10
20
30
40
50
% of diameter
C
relative Piezo2 mRNA level (%)
150
100
50
0
**
negative siRNA
Piezo2 siRNA
D
percentage of DPA neurons (%)
100
80
60
40
20
0
NR
nRI
RI
negative siRNA
Piezo2 siRNA
E
(μm)
3
0
τ = 5.82 ms
τ = 4.01 ms
400 pA
30 ms
negative siRNA
Piezo2 siRNA
F
maximal RI current (nA)
-2.5
-2.0
-1.5
-1.0
-0.5
0
negative siRNA
Piezo2 siRNA

Fig. 10. Pharmacological and genetic validation of Piezo2 currents in DPA neurons

A) Representative RI currents before (gray line), during (black line) and after washout (dotted line) of 30 μM ruthenium red (RR). **B)** Plot of RI current amplitude increase by incremental steps of mechanical stimulation before and during ruthenium red application (Two-way repeated-measures ANOVA followed by Bonferroni's post test, $p < 0.0001$). The probe was advanced to the half diameter of the recorded neuron. **C)** Comparison of Piezo2 mRNA level after negative or Piezo2 siRNA transfection. **D)** Classification of negative or Piezo2 siRNA transfected DPA neurons based on the decay time constant of mechanically evoked inward current. **E)** Representative RI currents from negative or Piezo2 siRNA transfected DPA neurons. **F)** Average maximal amplitude of RI currents from negative or Piezo2 siRNA transfected DPA neurons (Student's t test, $p > 0.05$).

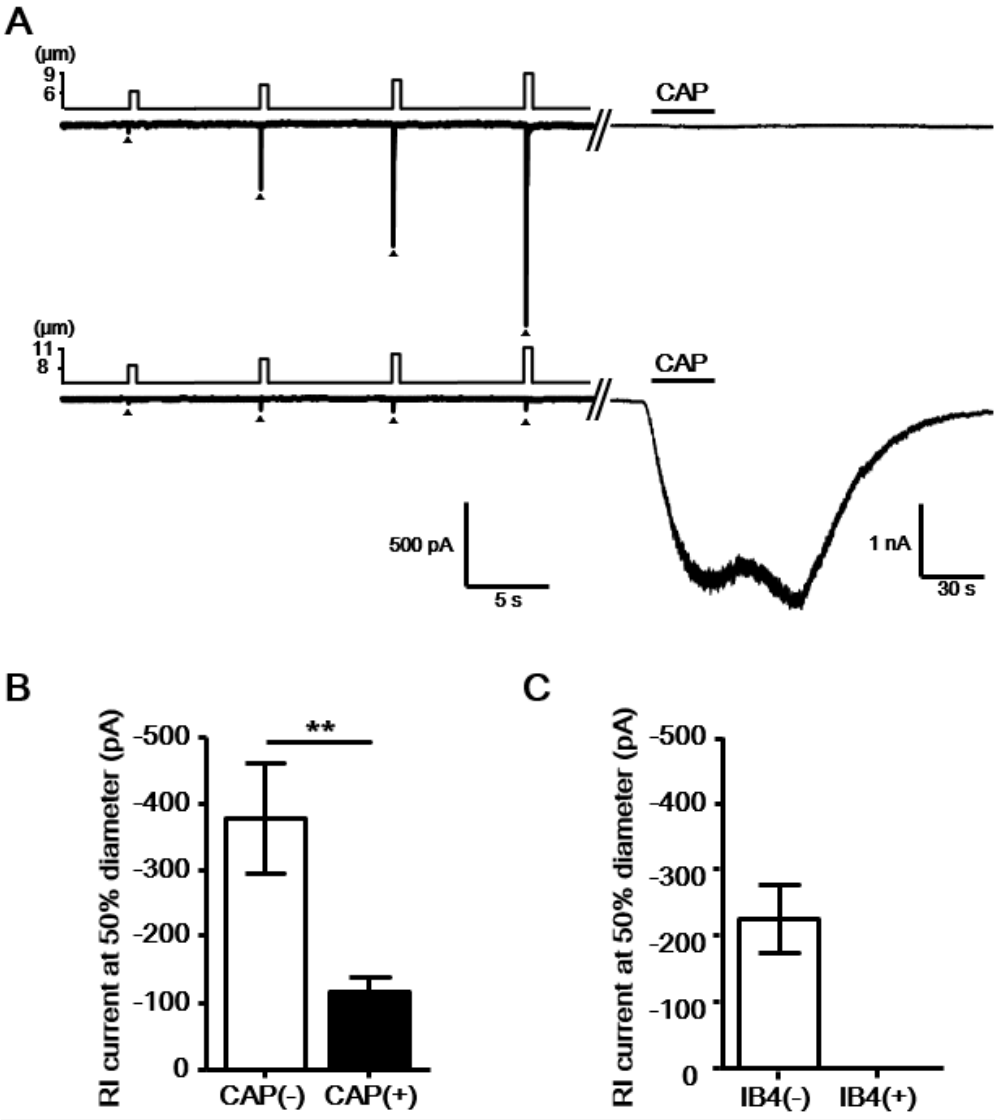

A
(μm)
9
6
CAP
(μm)
11
8
CAP
500 pA
5 s
1 nA
30 s
B
RI current at 50% diameter (pA)
-500
-400
-300
-200
-100
0
**
CAP(-)
CAP(+)
C
RI current at 50% diameter (pA)
-500
-400
-300
-200
-100
0
IB4(-)
IB4(+)

Fig. 11. Distinctive patterns of RI currents according to capsaicin sensitivity and IB4 positivity

A) RI currents evoked by a series of 1 μm incremental steps towards the DPA neurons being recorded, with a decay time constant less than 10 ms (Black arrowheads). DPA neurons showing Piezo2-like current were further tested for sensitivity to capsaicin (CAP, 500 nM). The upper panel is a representative trace from a DPA neuron which exhibited a series of RI currents in response to mechanical stimulation, but was insensitive to capsaicin. The lower panel is a representative trace from a DPA neuron which exhibited a series of RI currents in response to mechanical stimulation and was also sensitive to capsaicin. **B)** RI current amplitudes from capsaicin-sensitive or -insensitive DPA neurons. The probe was advanced to the half diameter of the recorded neuron (Student's t test, $p = 0.0094$). **C)** RI current amplitudes from IB4-negative or -positive DPA neurons. The probe was advanced to the half diameter of the recorded neuron.

DISCUSSION

The mechanical sensitivity in dental primary afferent system is elicited by mechanoreceptors in the tooth nerve terminal. In my study, I investigated Piezo mechanosensitive ion channels expressed in dental primary afferent (DPA) neurons. Single-cell RT-PCR confirmed that Piezo1 is not expressed in DPA neurons. On the other hand, Piezo2 mRNA is expressed in of 71% (n = 120/170) DPA neurons. Patch clamp recording also supports that Piezo2 is critical for the mechanotransduction of DPA neurons.

This is the first report identifying the mechanosensitive DPA neurons, the ever searched functional phenotype since the proposal of hydrodynamic theory (Brännström and Åström 1964). By using whole cell patch clamp recording and siRNA mediated-Piezo2 knockdown, I have identified that the majority of mechanosensitive DPA neurons produce RI currents in response to mechanical stimulation, which mainly seem attributable to Piezo2 expression. My results suggest that Piezo2 mediate mechanical transduction in DPA neurons, especially in medium- to large-sized neurons which contain nociceptive neuropeptides such as CGRP. This is consistent with my functional data showing Piezo2-like, RI currents exclusively in IB4-negative DPA neurons. Indeed, larger RI currents were more consistently evoked in capsaicin-insensitive DPA neurons than capsaicin-sensitive neurons.

To determine the neurochemical properties of Piezo2-positive DPA neurons, I characterized Piezo2 mRNA-positive DPA neurons with a range of neuronal markers: TRPV1, Nav1.8, CGRP, IB4, peripherin and NF200. TRPV1 is the receptor ion channel for noxious heat and pungent chemical capsaicin, and is expressed exclusively in nociceptors with lightly myelinated Aδ-fibers or unmyelinated C-fibers (Caterina and Julius 2001; Tal 1984). Nav1.8 participates in TTX-resistant action potential transduction in nociceptors (Abrahamsen et al. 2008). The expression of CGRP and IB4 indicate peptidergic and non-peptidergic nociceptors, respectively (Stucky and Lewin 1999). CGRP has been suggested as a potential mediator for dentin hypersensitivity, as CGRP-positive DPA neurons are mostly medium- to large-sized and NF200-positive, in contrast to CGRP-positive DRG neurons, which are mostly small-sized and NF200-negative (Fried, Sessle and Devor 2011; McCarthy and Lawson 1990; Pan et al. 2003; Vang et al. 2012). Peripherin is mostly expressed in small-sized, unmyelinated C-fiber nociceptors, whereas NF200 is generally expressed in large-sized non-nociceptors with myelinated, fast conducting A-fibers (Goldstein, House and Gainer 1991; Lawson et al. 1993).

My results on the mRNA expression of TRPV1 (44%) and Nav1.8 (69%), and IB4 (18%) positivity in DPA neurons is similar to previous findings (Kim et al. 2011; Paik et al. 2010; Pan et al. 2003). As my results showed that Piezo2 mRNA-positive DPA neurons are mostly medium- to large-sized, NF200 positive, and IB4 negative, it seems clear that they are rather functionally distinct from the small-

sized, unmyelinated DPA neurons which have been conventionally termed 'nociceptors' (Stucky et al. 2009). On the other hand, the high co-expression of Piezo2 and CGRP mRNA raises the possibility that these neurons could represent 'algoneurons', the putative mechanosensitive fibers that transduce weak mechanical stimuli into pain signals (Fried, Sessle and Devor 2011). The actual CGRP contents in Piezo2-positive DPA neurons, and whether this neuropeptide is released by mechanical stimulation, should be investigated further.

In the present study, the majority of Piezo2 mRNA-positive DPA neurons were positive for NF200, which may seem controversial as 89% of Piezo2 mRNA-positive neurons also co-express Nav1.8 (Abrahamsen et al. 2008). However, a recent paper have reported that Nav1.8 positive and NF200 positive/large sized sensory neurons are not discrete, but in fact overlapping populations (Ho and O'Leary 2011). Another recent study has also found that 40% of NF200 positive-DRG neurons co-express Nav1.8, with these neurons innervating Meissner corpuscles and hair follicles, thus indicating Nav1.8 expression among A-beta fibers (Shields et al. 2012). My results are consistent with these studies, suggesting this might be also the case in DPA neurons.

As above, my scRT-PCR experiments enabled us to characterize Piezo2 mRNA-positive DPA neurons by their size or molecular markers. In situ hybridization results obtained from mice also showed similar pattern of Piezo2 mRNA in DPA neurons according to cell size. I must caution, however, that the

markers for nociceptive neurons used here may have different expression profiles between rat and mouse.

The decay kinetics of mechanosensitive currents revealed Piezo2 expression in nearly 40% of DPA neurons. Previous studies also observed mechanosensitive RI currents in 21~44% of DRG neurons, which was mainly attributable to Piezo2 (Coste et al. 2010; Ranade et al. 2014b). These studies have also observed RI currents mostly in medium- to large-sized DRG neurons. Interestingly, the percentage of mechanosensitive DPA neurons with RI currents was higher than the population showing mechanosensitive currents with a slower decay kinetics (Coste et al. 2010; Ranade et al. 2014b), implying the physiological importance of Piezo2 expression in pulpal mechanosensory function. I was able to compare the expression level of Piezo2 mRNA and Piezo2-like currents in DPA neurons, which accounted for 71% and 40% of inspected DPA neurons, respectively. The presence of Piezo2 mRNA in 37.5% of mechanically insensitive DPA neurons reveals functionally silent Piezo2 mRNA, as confirmed by combining whole cell patch clamp and scRT-PCR analysis. Whether quiescent Piezo2 mRNA gains function during pathological changes of the pulp is yet to be found.

When RI currents were quantified according to cell diameter, the recorded currents were quite divergent in size, ranging from a few pA to > 3 nA. RI currents of nanoampere sizes were seen mostly in medium-to large-sized DPA neurons. These large RI currents were not observed after Piezo2 siRNA delivery, indicating these currents are derived from Piezo2 activation. RI currents relatively small in

amplitude (typically less than -100 pA when maximally induced) were also observed, regardless of cell size. The smaller and less mechanosensitive RI currents persisted even after siRNA mediated Piezo2 knockdown, consistent with previous studies which described small RI currents in Piezo2 knockout mice (Florez-Paz et al. 2016; Ranade et al. 2014b). The identity of the mechanoreceptor(s) underlying the residual RI currents evoked after Piezo2 knockdown in DPA neurons remains to be identified.

Functional characterization of DPA neurons by their IB4 positivity and capsaicin sensitivity revealed two features. First, RI currents were observed only in IB4-negative DPA neurons, which is consistent with the high expression of CGRP mRNA in Piezo2 mRNA-positive population. Second, the RI currents were significantly larger in capsaicin-insensitive DPA neurons. These two findings are also described in a previous report on DRG neurons in which RI currents were observed mostly in IB4-negative, capsaicin-insensitive neurons (Drew, Wood and Cesare 2002). Moreover, as it has been reported that only few TRPV1-positive DPA neurons co-express IB4, Piezo2-positive DPA neurons might be mostly IB4-negative, regardless of their capsaicin sensitivity (Vang et al. 2012). Based on these observations, Piezo2-positive DPA neurons are likely to be functionally distinct from thermosensitive, non-peptidergic DPA neurons, which are generally small-sized in diameter, unmyelinated and IB4-positive (Park et al. 2006).

In conclusion, I have confirmed that subpopulation of DPA neurons are indeed mechanically sensitive, and provide evidence suggesting that Piezo2

contributes in part to the mechanical responsiveness of DPA neurons. Piezo2-positive DPA neurons are likely to be medium- to large-sized with myelinated A-fibers, possibly mediating nociceptive peptidergic transmission when activated. Whether these properties reflect the function of 'algoneurons', the putative low threshold mechanoreceptors among DPA neurons which are paradoxically engaged in nociception, remains to be investigated (Fried, Sessle and Devor 2011; Ranade et al. 2014b). Further confirmation on whether Piezo2 activation can trigger action potential firings in DPA neurons, and if these Piezo2-positive populations do release nociceptive neurotransmitters will be helpful in advancing our understanding regarding dental nociception.

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국문초록

치수 구심성 신경에서 기계자극민감성 이온 채널의 발현 분석

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치성 통증의 발생을 설명하기 위해 유체역학설이 대두된 이래, 상아세관에서 발생하는 유체의 흐름에 반응하는 기계 자극 민감성 이온

채널이 치수 구심성 뉴런에 존재할 것으로 제안되었다. Piezo2 는 기계자극에 반응성을 보이며 빠르게 비활성화 되는 이온 채널로 척수 신경절 뉴런에서 발견되었으며 최근 촉각 전달을 중재하는 것으로 밝혀졌다. 이 연구에서, 치수 구심성 뉴런의 일부가 기계 자극 민감성을 나타낼 것으로 가정하고 치수 구심성 뉴런에서 Piezo2 의 messenger RNA 와 관련된 전기적 활성을 탐색하였다.

Piezo2 와 관련된 분자적/전기적 특성을 확인하기 위해 in situ hybridization, 단일 세포 역전사 중합 효소 연쇄 반응, 패치 클램프 기록법을 사용하였다.

Piezo2 mRNA 는 중형 내지 대형 치수 구심성 뉴런에서 주로 발견되었으며, 이들 중 대다수는 Nav1.8, CGRP 및 NF200 에 대해 양성이었지만 소수는 IB4 및 peripherin 에 대해 양성이었다. 패치 클램프를 통하여 치수 구심성 뉴런의 일부에서 기계적 자극에 의해 유발된 Piezo2 치수 구심성 뉴런의 일부에서 기계적 자극에 의해 유발된 Piezo2 유사 전류 (RI 전류 : rapidly inactivating)를 확인하였다. 이러한 RI 전류는 Piezo2 를 차단하는 것으로 알려진 화합물인 ruthenium red 에 의해 억제되었으며 small-interference RNA 에 의한 Piezo2 knockdown 후에는 관찰되지 않았다. Piezo2 유사 전류는 IB4 음성 치수 구심성 뉴런에서 거의 독점적으로 관찰되었다. 또한 Piezo2 유사 전류는 캡사이신에 민감하지 않은 뉴런에서 캡사이신에 민감한 군보다 크게 나타났다.

따라서 치수 구심성 뉴런은 기계 자극 민감성을 보이며 이러한 현상의 일부는 Piezo2 이온 채널에 의해 매개되는 것으로 보인다. 또한 Piezo2 양성 치수 구심성 뉴런은 대부분 통각성 펩타이드 신호전달물질을 포함하는 myelinated A-fiber 를 가진 중대형 뉴런의 특성을 나타내는 것으로 확인되었다.

주요어 : 치수 구심성 신경, 치수, 상아모세포, 기계 자극 민감성 이온 채널, Piezo1 채널, Piezo2 채널.

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