TRPV1 expressing afferents mediate orthodontic tooth movement via Piezo2

2022 Research Aid Awards (RAA)

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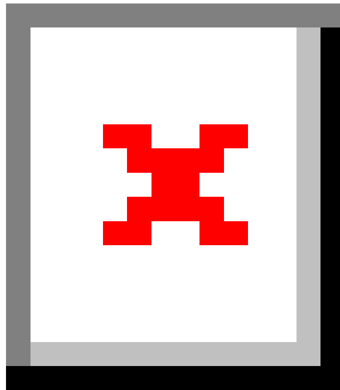

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TRPV1 expressing afferents mediate orthodontic tooth movement via Piezo2

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Institution
The Ohio State University College of Dentistry

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Abstract
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Abstract
Orthodontic force therapeutically induces mechanical stimuli and inflammation in the periodontium, which is inevitably accompanied by pain. In the past one hundred year, it has been well known that aseptic inflammation regulates biological process of bone resorption during orthodontic tooth movement (OTM). Many studies considered orthodontic pain is induced by orthodontic force directly, and it is amplified by following inflammatory responses such as the release of neurogenic and pro-inflammatory mediators. Other research elucidate that neurological pain can reprogram genes in T-cells and modulate tissue inflammation and bone resorption. In periodontium, a quarter of sensory nerves contain both transient receptor potential vanilloid 1 (TRPV1), a receptor for capsaicin (an active component of chili peppers), and Piezo2 (a mechanosensitive ion channel). Our preliminary finding showed that TRPV1 expression increased with application of orthodontic force. Pharmacologically blocking out TRPV1 response decreased tooth movement. Although our data imply an important role of peptidergic afferents (e.g., TRPV1-contaning neuron) in orthodontia, it is unclear how mechanical forces trigger TRPV1 expression. Therefore, we hypothesize that orthodontic pressure can furnish with discomfort (e.g., sourness and pain) via Piezo2 activation, which then promotes TRPV1 expression and orthodontic force-induced alveolar bone remodeling. This project is significant because it promises to elucidate enigmatic linkage between orthodontic force induced pain and alveolar bone remodeling by demonstrating the role of sensory nerves and mechanosensitive ion channel Piezo2 in initiating orthodontic tooth movement. The findings may make it possible to develop novel therapeutic strategies for accelerated tooth movement by manipulation of sensory afferents.

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Review: Mechanosensitive ion channels initiate tooth movement: from force to tooth movement

1. Introduction

The main objective of orthodontic treatment is to correct malocclusion by moving teeth inside alveolar bone. There are two important components during tooth movement: Force and Bone Remodeling. Force from orthodontic appliances is delivered to the tooth through brackets on each tooth. The tooth and periodontium around the tooth react to the force through mechanosensitive ion channels on cells, which cause Ca^{2+} influx and convert mechanical signals to electrical signals and signaling cascades that ultimately lead to bone remodeling and orthodontic tooth movement. This review summarizes the current understanding of mechanosensitive ion channels in bone remodeling and its implication in orthodontic tooth movement.

2. Mechanosensitive Piezo1 and Piezo2 ion channels

In 2010, Piezo1 and Piezo2 were emerging as important mediators of various aspects of mechanotransduction. These channels convert applied force into electrochemical signals critical for proprioception, sensation of touch, as well as mechanical pain [1]. Piezo1 was highly expressed in various non-neuronal tissues, such as vasculature, bone, heart, and has been shown to sense different mechanical stresses such as compression and stretch. On the other hand, Piezo2 showed exclusive expression in sensory neurons, and was related with touch sensation and mechanical pain [1, 2]. However, both Piezo1 and Piezo2 affected the skeletal system. First,

Piezo1 deficiency hindered bone formation and caused atypical reactions to external force [3-6]. Moreover, emerging evidence indicates that Piezo1 and Piezo2 participate in regulating signaling transduction in periodontal bone remodeling and orthodontic tooth movement (OTM) [7-10].

3. Mechanosensitive Piezo1/2 ion channels in tooth movement

3.1. Bone remodeling in tooth movement

Bone remodeling is an important biological metabolism to maintain healthy bone density and homeostasis and to regulate orthodontic tooth movement. Alveolar bone is under the most active bone remodeling among the entire skeletal system [11-14]. According to the pressure-tension theory, alveolar bone coupled remodeling during orthodontic treatments is orchestrated by the activities of osteoclasts, osteoblasts, and osteocytes in periodontal ligament space and alveolar bone, after force applied to teeth by archwire. Osteoclasts are specialized cells originating from hematopoietic progenitors, which can resorb the host bone tissue [15, 16]. Osteoblasts are mononucleated and specialized cells originating from mesenchymal stem cells (MSCs) are the primary responsible for alveolar bone apposition [17]. Osteocytes are derived from functional osteoblasts and the dominant cells embedded in the mineralized bone in the process of bone apposition [17].

On the tension side, the periodontium, including the periodontal ligaments, alveolar bone, and cementum, undergoes bone deposition. Osteoblasts originating from MSCs form the osteoid or type I collagen matrix, following mineralization [18]. Meanwhile, alveolar bone resorption takes place on the compression side due to osteoclastic activity, resulting in irregular cavities in bone which are filled by new bone from osteoblast activity [19]. Orthodontic forces lead to the

increase of blood vascular permeability and disarrangement of tissues in the periodontal ligament space. Subsequently, blood flow and periodontal tissue adapt to the compression force. If they fail, tissue necrosis and hyalinization will occur [20]. Force magnitude is deemed to be related to the origination of osteoclastogenesis and types of bone resorption. Applying light force leads to front resorption, hematopoietic progenitors recruited from blood vessel in periodontal ligament space, cell and tissue preservation. Applying heavy force causes undermining bone resorption and hyalinization, cell death and tissue necrosis as well as cell-free periodontal ligaments and adjacent alveolar bone zones, leading to delayed bone resorption. In both human studies and animal studies, heavy force causes more discomfort and pain compared with light force [21-23].

3.2. Mechanosensitive Piezo1/2 ion channels in Osteoblast

Osteoblast lineage cells, including osteoblast progenitors, osteoblasts, and osteocytes, are differentiated from mesenchymal stem cells (MSCs). These cells express Piezo1. In newborn mice, loss of either Piezo1 or both Piezo1 and Piezo2 in MSCs or osteoblast progenitor cells showed suppression of osteoblast differentiation and increased bone resorption, resulted in multiple spontaneous bone fractures in newborn mice [6]. Moreover, Piezo1 and Piezo2 knockout mice were less susceptible to further bone loss in bone development and homeostasis due to lack of loading [6]. When hydrostatic pressure is applied on MSCs, Piezo1 is activated and plays a role for cell fate determination of MSCs either osteoblast differentiation or adipocyte differentiation by regulating bone morphogenetic protein 2 (BMP2) expression [24]. On the other hand, Activation of Piezo1 by its agonist, Yoda1, induced Ca^{2+} response and activated cationic currents in osteoblastic cells, followed by reduced the proliferation, which was reversed by knockdown of Piezo1 [25]. Furthermore, the C-terminus of PIEZO1, which contains the R-

Ras binding domain, plays an essential role in Ca^{2+} influx and activation of the ERK1/2 signaling pathway, suggesting that this domain is crucial for the mechanotransduction of osteoblastic differentiation in MSCs [26]. At the end of adolescence, mechanical force actives Piezo1 in osteoblasts, enhancing bone mineralization [27]. Additionally, human studies have identified the SNP rs62048221 in the enhancer region of the PIEZO1 gene, which significantly influences bone mineral density, in human MSCs [28].

3.3. Mechanosensitive Piezo1/2 ion channels in Osteocyte

Osteocytes, located within the bone matrix, detect changes in mechanical load and produce signals that regulate osteoblasts to modify bone formation. Conditional *Piezo1* knockout in osteoblasts and osteocytes in mice showed reduced bone mass and strength, meanwhile, administration of a Piezo1 agonist to adult mice increased bone mass, replicating the effects of mechanical loading [3]. Piezo1, located on osteocytes, contributes to bone formation through specific biological pathways. 1). Piezo1 on osteocytes aids in bone formation by following pathways: 1). In osteocytes, the activation of the Piezo1-Akt pathway is necessary for mechanical stretch-induced decreased the expression of Sost (encoding Sclerostin), therefore resulting in enhancing the genesis of osteoblasts and bone formation [29]. 2). *Piezo1*-mediated stress promotes the expression of Osteoprotegerin (OPG) and suppresses the expression of Nuclear Factor-Kappa-B Ligand (RANKL) via NOTCH3 in osteocytes [30]. These pathways involve the translation of mechanical stimuli into cellular signals that regulate activities of both osteoblasts and osteoclasts, as well as the process of bone remodeling.

3.4. Mechanosensitive Piezo1/2 ion channels in Osteoclast

In contrast to the osteoblasts lineage, osteoclasts are differentiated from hematopoietic progenitors. Piezo1 plays a role in crosstalk between osteoblast and osteoclast in response to mechanical loading. For example, osteoblastic cells regulate the YAP-dependent expression of type II and IX collagens, which control osteoclast differentiation, to maintain bone homeostasis [5]. However, mice display normal skeletal phenotype if only targeted deletion of Piezo1 in osteoclasts using Ctsk-Cre mice [5], Lyz2-Cre mice [31]. These results from in vivo experiments are consistent with the in vitro experiment in osteoclast-like cell line RAW264.7 [4].

3.5. Mechanosensitive Piezo1/2 ion channels in chondrocytes

There are two osteogenic pathways: intramembranous ossification and endochondral ossification. The role of Piezo1 in intramembranous ossification was not very clear, but Piezo1 affects in the process of endochondral bone formation, which is also important for growth modification, such as Herbst and twin blocks. In the process of endochondral ossification, Piezo1 plays a critical role in growth plate chondrocytes to ensure the formation of trabecular bone since targeted deletion of Piezo1 in chondrocytes (Col2a1Cre) showed osteoporosis with fractures, and impaired formation of the spongiosa with atypical osteoblast morphology [31]. Furthermore, chondrocytes could perceive and distinguish the mechanical loading using different mechanosensitive ion channels, TRPV4 is pivotal in chondrocytes reacted to physiological strain levels (3% and 8%), while Piezo2 is essential in chondrocyte response to injurious strain levels (18%) [32].

3.6. Mechanosensitive Piezo1/2 ion channels in Neurons

Piezo2, known as an essential mechanotransduction channel in sensory nerve system with high expression, affects sensation of touch, mechanical pain, and tactile allodynia [33-36].

Conditional deletion of Piezo2 in central or peripheral afferents decreased the touch sensation, but not mechanical pain and tactile allodynia in mice [33, 34, 37, 38]. Interestingly, selective deletion of Piezo2 in neurons through *PValb-Cre,* but not *Prx1-Cre*, *Col1a1-Cre* or *Col2a1-Cre,* causes spine malalignment and abnormal joints, which might result from the impaired skeletal muscle functions through the lack of the sense of proprioceptive in motor neurons [39]. This phenotype in mice closely resembles those findings in the diseases associated with PIEZO2 mutations in human [39].

3.7. The role of Mechanosensitive Piezo1/2 ion channels in tooth movement

The periodontal ligament (PDL), a thin layer of fibrous connective tissue between the tooth and the alveolar bone, is crucial for the development, functioning, and regeneration of periodontium, including alveolar bone. Piezo1/2 in PDL cells (PDLCs) are keys in maintaining the homeostasis of periodontal tissue [40, 41].

As key mechanotransducers, Piezo1 is expressed increasingly in PDLCs under both tensile and compressive stress through different signaling pathways [42, 43]. Under tensile stress, Piezo1 expression in PDLCs significantly increases through the ERK signaling pathway [43]. Meanwhile, under compressive stress, both the expression of Piezo1 and osteoclastogenesis markers in PDLCs are increased, which is suppressed by grammostola mechanotoxin 4 (GsMTx4), a Piezo1 inhibitor, via nuclear factor kappa B (NF-kB) signaling pathway [42]. The expression of Piezo1 in PDLCs has been showed under various mechanical stresses, including physiological and externally.

During a lifetime, teeth and periodontal tissues are exposed to physiological stress, such as occlusal forces during biting, chewing, and swallowing. If lack of occlusal force occurs due to

missing teeth, unbalanced bone remodeling results in a net alveolar bone loss around the region with missing teeth. Furthermore, adults with hyperdivergent mandibles tend to have lower maximum bite and thinner cortical bone than in hypodivergent subjects [44, 45]. In alveolar bone, the periodontal ligaments plays an essential role in mechanotransduction by converting physical forces into the signaling circuit Piezo1/Ca^{2+}/HIF-1α/SLIT3, which promotes type H angiogenesis and OSX+ cell-related osteogenensis, playing a significant role in maintaining the alveolar bone density and volume [46]. These findings address the issue that guided bone regeneration membranes, which are used to block fibroblast penetration and facilitate bone defect repair by osteoblasts, show limited bone regeneration due to the lack of bone induction and angiogenic capacities. A Yoda1-loading bilayer membrane using electrospinning technology: the inner layer releases Yoda1, Piezo1 agonist, to promote bone regeneration, the outer layer blocks fibroblast infiltration and immune response [47]. Furthermore, Yoda1 affects angiogenesis and osteogenesis to accelerate bone regeneration [47].

Orthodontic tooth movement (OTM) is a distinct process, induced by external mechanical loading, featured by bone and periodontal remodeling. On the tension side, the expression of Piezo1 and the makers related to osteogenesis are dramatically increased, whereas inhibition of Piezo1 by GsMTx4 slow down OTM [9]. Interestingly, after exposure to mechanical loading in primary human PDLCs in vitro study, the expression of Piezo1 and markers for osteoclastogenesis, such as receptor activator of nuclear kB Ligand (RANKL) and cyclooxygenase-2 (Cox-2), were significantly increased [42]. Furthermore, GsMTx4, a Piezo1 inhibitor, blocked osteoclastogenesis [42], suggesting Piezo1 contributes to the mechanical stress-induced osteoclastogenesis. Additionally, under compression force, Piezo1 on the

membrane of human periodontal ligament fibroblasts is activated, leading to the release of ATP through intracellular Ca2+-dependent exocytosis and channels that are permeable to ATP [48]. Piezo1 also plays a role in macrophages infiltrate in PDL via Piezo1-AKT/GSK3b signaling-Cyclin D1 (Ccnd1) axis during tooth movement [8], and mediating both the osteogenesis and osteoclastic activities on the tension side during OTM [9, 49, 50]. In animal tooth movement models, however, the function of Piezo1 is controversial. In rats, orthodontic tooth movement was modestly reduced by the local injection of GsMTx4 into alveolar bone [50], whereas tooth movement was not altered in conditional knock out of Piezo1 mineralized tissue cells with Dmp1-cre [51]. However, Piezo2, when expressed in periodontal afferents, regulates tooth movement remains to be investigated.

4. **Clinical perspectives**

In the biologic basis of OTM, orthodontists believed the Piezoelectricity, which is a phenomenon, in many crystalline materials, involves a deformation of the crystal structure due to external force, leading to electric current as electrons are displaced from one part of crystal lattice to another. Both inorganic crystals, such as bone, and organic crystals, such as collagen crystals in the PDL, can be piezoelectric. This piezoelectricity might initiate orthodontic tooth movement since it happens immediately after force loading. Then local hypoxia in the compression side of PDL, which initiates an aseptic inflammatory cascade culminating, leads to cellular differentiation and ultimately tooth movement. However, there are two shortages in this biologic therapy.

First, piezoelectricity is a phenomenon, which is a hypothesis without confirmation by experiments. The recent discovery and identification of mechanosensitive ion channel, Piezo1/2 by Nobel laureate Ardem Patapoutian will greatly aid to explore our knowledge of how force initiates tooth movement. A wealth of in vitro and in vivo studies in skeletal system have further clarified our understanding of the transition from force to bone remodeling during orthodontic movement. *In vivo* studies using mouse genetic models could pinpoint crucial Piezo1/2 signaling pathways in specific cells during osteogenesis and osteoclastogenesis at the tension and compression sites in PDL in OTM. Additionally, inflammation causes bone resorption on the compression side in OTM and displays limited affect bone formation via osteogenesis on the tension side. Recent research about Piezo1/2 in MSCs, PDLCs, osteoblastic line cells, as well as osteoclasts has broadened our insight into the various capabilities in both osteogenesis and osteoclastogenesis.

During orthodontic treatment, both clinicians and patients want to achieve excellent outcomes within a short treatment duration. Accelerating tooth movement became one of the most popular topics involves numerous studies and patents of methods and devices. Non-surgical interventions, such as light vibration, do not cause pain but fail to accelerate tooth movement [52, 53]. The only method with high evidence to accelerate tooth movement is corticotomy through induction of local osteoclastogenesis and macrophage infiltration [54], which causes inflammation, pain, discomfort, and functional impairments [55]. Selective deletion of Piezo1 on osteoblasts/cementoblasts, osteocytes/cementocytes and odontoblasts does not change the morphology of skull and tooth movement but causes significant bone loss [51]. This finding also clarifies why Low-frequency vibration (30 Hz) fails to accelerate tooth movement, despite *in*

vitro studies indicating its potential to boost osteoblastic activity [56]. Additionally, Piezo2 is expressed in transient receptor potential vanilloid subtype 1 (TRPV1)-expressing nociceptors, which in the current international association for the study of pain (IASP) is defined as "a high-threshold sensory receptor of the peripheral somatosensory nervous system that is capable of transducing and encoding noxious stimuli." The term refers to the nerve endings that initiate the transduction of noxious stimuli rather than molecule and mediate mechanical pain cornea, visceral mechanical hypersensitivity, and mechanical hyperalgesia in knee joint osteoarthritis [57-59]. TRPV1-expressing afferents enhance bone destruction in periodontitis by promoting a hyperactive host response in the periodontium. Therefore, it is plausible that Piezo2 in TRPV1-expressing TG neurons regulate OTM and orthodontic pain, which will be determined in this study.

7. Conclusion

Despite decades of studies, the details of how force initiates orthodontic tooth movement remain unclear. Particularly, the ongoing knowledge gap and challenges remains substantial in mechanistic understanding of the mechanosensitive ion channels, Piezo1/2, during tooth movement. Better understanding of the fundamental mechanisms of mechanosensitive ion channels may improve orthodontic treatment by developing new methods to accelerate orthodontic tooth movement.

METHODS AND MATERIALS

Collect neurons from trigeminal ganglion (TG) and dorsal root ganglion (DRG)

Both 3 to 6 weeks-old male and female *C57BL/6* mice were used for TG and DRG collection. All surgical instruments were sterilized before procedure. Mice were euthanized by exposing to carbon dioxide (CO_2). Head and lumbar spine were isolated by dissection scissors. Before collecting TG and DRG, tissues were placed on the ice.

For TG collection, skull was cut from Lambda to Bregma by Fisherbrand™ High Precision Dissecting Micro Scissors. Subsequently, skull disk and brain were removed to expose the trigeminal ganglion at the base of the skull. Each TG has three branches. The branches were snapped at the point where they exit from the skull. Then micro-scissors were slid under and along the TGs to separate the TG from connective tissue. Excelta™ Titanium Alloy Tweezers were used to gently lift the TGs out and place them into a 35-mm culture dish with 2 mL ice-cold serum-free medium, containing 10% fetal bovine serum, 100 mM sodium pyruvate, and 1x penicillin/streptomycin in 1x DMEM-F12 (ThermoFisher Scientific, #12634010), the on the ice.

For DRG collection, two cuts along the sides of the spinal column (left and right) were made by micro-scissors to remove the dorsal portion of the vertebrae and to expose spinal cord. Subsequently, spinal cord was removed by micro tweezers. Lumbar DRGs are in the discs between the vertebrae. Individual DRGs were meticulously isolated by micro tweezers to clasp and lift the axon bundles projecting distally, which are found on the lateral side of the DRG. Care must be taken not to damage the DRG with the micro tweezers. Bilateral lumbar DRG (L1-L6) were collected by into the same culture dish with TG.

Large pool pf TGs and DRGs were collected from 4 mice each time to keep the duration of the procedure within one hour.

Primary culture of neurons

Culture medium, which contains 10% fetal bovine serum (ThermoFisher Scientific, #26140079), 100 mM sodium pyruvate (ThermoFisher Scientific, #11360070), and 1x penicillin/streptomycin (ThermoFisher Scientific, #15140122) in 1x DMEM-F12 (#12634010), was prepared in advance. Corning™ BioCoat™ Poly-D-Lysine Multiwell Plates (Corning™356414) or coated stretch chamber (SC-0044) were used. Coating protocol for stretch chambers: The stretch chambers were autoclaved at 121°C for 20 minutes, then they were coated with 200 µg/mL poly-L-lysine at 37°C for 4 hours, then the chambers were rinsed twice with serum-free culture fluid. Culture dishes with 2 ml culture medium were placed in a 37 °C CO_2 incubator for least 1 hour before experiment. The 35-mm culture dish, containing a large pool of TG and DRG, was transferred into a hood after the outside of the dish had been cleaned with 75% ethanol. Before being transferred to a new 35 mm culture dish, TGs and DRGs were washed with serum-free medium using a pipette. The new 35 mm culture dish contained 2 ml of collagenase type IA (ThermoFisher Scientific, #17100017, 1 mg/ml in serum-free medium, sterilized by passing through a 0.22 µm syringe filter) using sterile tweezers. TG and DRG were digested in the collagenase solution at 37 °C in a CO2 incubator for 15 minutes, and then further digested on a lab shaker at room temperature for 30 minutes. After removing the collagenase solution, TG and DRG were washed with 2ml Hank's balanced salt solution (HBSS) (ThermoFisher Scientific, #88284). 2 ml pre-warmed 0.05% trypsin-EDTA (ThermoFisher Scientific, #25300054) was added to the 35 mm dish containing TG and DRG. They were then digested at 37 °C in a CO2

incubator for 15 minutes, followed by further digestion on a lab shaker at room temperature for 30 minutes. The 2 ml solution with TG and DRG was carefully transferred to a 15 ml centrifuge tube using a pipette, centrifuged at 290 x g at 4 °C for 5 minutes, after which the supernatant was discarded and replaced with another 2 ml of pre-warmed culture medium to resuspend the TG and DRG. The TG and DRG were carefully triturated using first a 1250 µl pipette and then a 200 µl pipette, approximately 20 times with each. The Poly-D-Lysine Multiwell Plate or poly-L-Lysine-coated stretch chamber was moved from the CO2 incubator to the hood, where the incubated culture medium was aspirated, and the dissociated cells were then seeded onto the coated dish. Neurons from 8 TGs and 48 DRGs, obtained from 4 mice, were seeded into four wells; there were approximately 5, 000 cells in one well of a 24-well plate in 2 ml growth medium containing αMEM (Life Technologies), 10% heat inactivated-FBS (Life Technologies), and 1X Glutamine-Penicillin–Streptomycin (Invitrogen) and were allowed to attach for 24 h in 5% CO2 and 37ºC. Half of the medium was carefully replaced every other day to avoid aspirate the neurons for 4 days.

CGRP and Substance P release of neuron cultures

For vehicle group and capsaicin groups, neurons from TG and DRG were cultured as described above in a 24 well plate (5,000 cells per well, 2 cm^2 per well) for 4 days. Media was aspirated and replaced with 200 µl fresh growth medium with vehicle or 1 µM capsaicin to stimulate neurons for 1 hour at 37 °C with 5% CO2. For mechanical stretching loading groups with or without the mechanosensitive channel inhibitor GsMTx4, neurons from TG and DRG were cultured as described above in coated stretch chamber (SC-0044) with 4 wells (10, 000 cells per

well, 4 cm^2 per well) for 4 days with 2 turns each day to increase the ratio by 1mm (equating to a 5% increase and 20% totally) for 1 hour at 37 °C with 5% CO2. Media was aspirated and replaced with 100 µl fresh growth medium with or without 50 µM GsMTx4 (Abcam), which was prepared in 0.1% dimethyl sulfoxide (DMSO). 50 µl of culture supernatant was collected for CGRP concentration analysis using a mouse CGRP EIA Kit (Invitrogen), and an additional 50 µl was collected for Substance P concentration analysis using a Substance P competitive EIA Kit (Invitrogen). In total, 200 µl of culture supernatant was collected in 4 days for CGRP and Substance P ELISA analysis. In the context of mechanical stretching loading groups, 2 ml the growth medium was meticulously replaced following the collection of the culture supernatant, except for day 4.

For each experimental condition, namely the vehicle group, capsaicin group, stretching loading group, stretching loading group treated with GsMTx4, 2 to 3 wells were allocated. This procedure was replicated 4 times, ensuring the robustness and reproducibility of the data.

ELISA was carried out utilizing a commercial kit, following the protocol provided by manufacturer, with some adjustments.

Statistical Analysis

All data are presented as mean ± SEM. The data were analyzed using Student's t tests and 1-way or 2-way ANOVA followed by post hoc assays.

RESULTS AND DISCUSSION

Previous studies have focused on the contribution of neuropeptides to orthodontic pain. A recent study revealed that systemic injection of exogenous SP accelerated orthodontic tooth movement in rat model.[60] In our research, the findings from knockout endogenous SP in mice reduced orthodontic tooth movement, indicating that SP promotes alveolar bone remodeling and accelerates orthodontic tooth movement.

To determine whether mechanical stretching loading can directly activate peptidergic Piezo2+ neurons, we treated cultured neurons with mechanical stretching loading. Interestingly, in our in vitro experiment, we found that mechanical stretching loading triggered significantly more CGRP and SP release compared to vehicle treated group (Fig. 4B-C). Under mechanical stretching loading, mechanosensitive channel inhibitor GsMTx4 reduced the release of CGRP but not SP, the reduction in CGRP release was similar to what was observed in vehicle group without mechanical stretching loading, indicating that Piezo2 contributes to the CGRP release more than SP induced by mechanical stretching loading (Fig. 4B-C). Capsaicin, the active component of chili peppers, is a prototypic TRPV1 ligand that activates nociceptors [61]. Capsaicin triggered robust CGRP and SP release in vitro (Fig. 4B-C). These results indicate that neurons can directly sense mechanical stretching loading and capsaicin to release SP and CGRP, but CGRP is more related to mechanical stretching loading compare to SP.

The results mentioned do not support a direct interaction between Piezo2+ neurons and Substance P, challenging the proposed pivotal role of TRPV1+ neurons in this process. There are a few causes leading to the results. First, in the in vitro experiment, cells were stretched, which

simulates the tension side rather than the compression side; however, in the in vivo experiments, we focused on the compression side. To overcome this limitation, we can use conditional knockout (cKO) of Piezo2 in SP-positive afferents in animal studies, or we can employ a device that produces compression force to replicate the in vitro study. Second, in the in vitro experiment, sample size is relatively small and ELISA is a very sensitive technology. Third, this in vitro experiment showed the importance of Piezo2 for releasing of CGRP, thus we can use CGRP knock out mice to test the function of CGRP during tooth movement. Last, GsMTx4 inhibits both Piezo1 and Piezo2, as well as other mechanosensitive ion channels, which could also effect the results. D- GsMTx4 would be a better choice to block Piezo2 only. Second, in the in vitro experiment, the sample size is relatively small, and ELISA is a very sensitive technology. To achieve more accurate results, we would repeat the experiments. Third, this in vitro experiment highlighted the importance of Piezo2 in the release of CGRP; therefore, we could use CGRP knockout mice to test the function of CGRP during tooth movement. Lastly, GsMTx4 inhibits both Piezo1 and Piezo2, as well as other mechanosensitive ion channels, which could also affect the results. D-GsMTx4 would be a better choice to specifically block Piezo2.

Orthodontic tooth movement represents a distinctive process of bone remodeling induced by mechanical loading.[62] Patients typically experience discomfort or mechanical pain immediately after the placement of an initial arch wire or a stiffer wire. This phenomenon might be one of the earliest biological reactions in orthodontic treatment. This novel study creates a fresh understanding of how tooth movement is initiated. In comparison to Piezo2, Piezo 1, another mechanically activated Ca^{2+} permeable channels predominantly expressed mainly in non-neuronal cells,[1, 63] has been extensively investigated in the context of orthodontic tooth

movement. Piezo1 expression and markers osteoclastogenesis-related markers were found to increase in PDL cells under compressive stress and on the tension side during OTM in an animal model. Additionally, the inhibition of Piezo1 by GsMTx4 demonstrated a decrease in the ability of PDL cells to induce osteoclastogeneisis in vitro and inhibited OTM in vivo, acting through the Piezo1–AKT–cyclin D1 axis.[8, 9, 42]

Most importantly, orthodontic tooth movement represents a distinctive process of bone remodeling induced by mechanical loading.[62] Patients typically experience discomfort or mechanical pain immediately after the placement of an initial arch wire or a stiffer wire. This phenomenon might be one of the earliest biological reactions in orthodontic treatment. This study implies a fresh understanding of how tooth movement may be initiated. In comparison to Piezo2, Piezo 1, another mechanically activated Ca^{2+} permeable channels predominantly expressed mainly in non-neuronal cells,[1, 63] has been extensively investigated in the context of orthodontic tooth movement. Piezo1 expression and markers osteoclastogenesis-related markers were found to increase in PDL cells under compressive stress and on the tension side during OTM in an animal model. Additionally, the inhibition of Piezo1 by GsMTx4 demonstrated a decrease in the ability of PDL cells to induce osteoclastogeneisis in vitro and inhibited approximately 30% OTM in vivo, acting through the Piezo1–AKT–cyclin D1 axis.[8, 9, 42]

The clinical relevance lies in the acceleration of tooth movement. As reported in clinical studies and systematic review, the use of light vibrational forces by AcceleDent Aura device demonstrated no difference in the rate of OTM and patient perception of pain.[53, 64] This outcome aligns with our study, suggesting that the force applied may not reach activation thresholds for Piezo2 in TRPV1 nociceptors. To explore this further, increasing the intensity and

frequency of the forces might be necessary. To validate this approach, additional studies ranging from bench experiments to pre-clinical investigations are required. This comprehensive research will provide a more thorough understanding of the impact of intensified forces on Piezo2 activation and its potential implications for accelerating tooth movement in clinical settings.

In conclusion, our study reveals, for the first time, that TPRV1+ neurons in the TG play a pivotal role in mediating tooth movement through the involvement of SP and Piezo2. This newly uncovered neuroskeletal mechanism holds significant implications for comprehending the dynamics of force, patients' perception and the associated bone turnover. It could potentially emerge as a groundbreaking theory in the field of tooth movement and serve as a promising therapeutic target for accelerating orthodontic treatment.

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Figure 1 Piezo2 senses mechanical stimuli to release CGRP and SP

A. groups and timeline.

B. Substance P releasing using ELISA. **$p<0.01$, ****$p<0.0001$ in ANOVA.

C. CGRP releasing using ELISA. **$p<0.01$, ****$p<0.0001$ in ANOVA.

A

	Day 0	Day 1	Day 2	Day 3	Day 4
Vehicle					+ (1h)
Capsaicin					+ (1h)
Mechanical loading +/- antagonist (5% each day, up to 20%)		+ (1h)	+ (1h)	+ (1h)	+ (1h)

B

SP ELISA

SP (pg/ml)
10000
1000
100
10
1
****
****
**
****
****
ns
vehicle
mechanical loading
mechanical loading + antagonist
capsaicin
groups

C

CGRP ELISA

CGRP (pg/ml)
150
100
50
0
ns
vehicle
force loading
force loading + antagonist
capsaicin
groups