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NEUROPHYSIOLOGY

Odontoblast TRPC5 channels signal cold pain in teeth

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Teeth are composed of many tissues, covered by an inflexible and obdurate enamel. Unlike most other tissues, teeth become extremely cold sensitive when inflamed. The mechanisms of this cold sensation are not understood. Here, we clarify the molecular and cellular components of the dental cold sensing system and show that sensory transduction of cold stimuli in teeth requires odontoblasts. TRPC5 is a cold sensor in healthy teeth and, with TRPA1, is sufficient for cold sensing. The odontoblast appears as the direct site of TRPC5 cold transduction and provides a mechanism for prolonged cold sensing via TRPC5's relative sensitivity to intracellular calcium and lack of desensitization. Our data provide concrete functional evidence that equipping odontoblasts with the cold-sensor TRPC5 expands traditional odontoblast functions and renders it a previously unknown integral cellular component of the dental cold sensing system.

INTRODUCTION

Insults to the tooth's dentin produces inflammation, most commonly during tooth decay. Dental caries is a chronic disease in which a bacterial biofilm on the tooth surface, in combination with fermentable carbohydrate substrates, causes demineralization and eventually tooth decay. Worldwide, 2.4 billion people have untreated caries in permanent teeth (1). Inflamed teeth are extremely cold sensitive, perceived as a short, sharp intense neuralgic pain (2). On the basis of the functional anatomy, in which ceramic-like enamel and dentin insulate nociceptive terminals from temperature changes (3), the tooth pulp's sensory plexus of Raschkow is widely accepted as mechano- and nociceptive. In Brännström's hydrodynamic or fluid movement theory, the transduction of thermal and other physical

stimuli to activate dentinal nerve endings has been attributed to a fluid dynamic–induced mechanosensory process. In this theory, dentinal microcanals (tubules) act as a hydraulic link between the physical stimulus and the nerve terminals, which are sited at the pulp-dentin boundary (fig. S1) (4). Functional experimental evidence for this theory is lacking.

The lack of functional evidence for cold sensing in teeth is unexpected given the progress in our understanding of molecular cold-sensing molecules (5). Certain transient receptor potential (TRP) ion channel subtypes are strongly activated by cooling, acting as molecular sensors in the skin and mucous membranes where they depolarize nerve terminals to elicit action potentials (5). In the skin, TRPM8 and TRPA1 act synergistically and represent the key sensors of environmental cooling as well as painful cold (5, 6). TRPM8 and TRPA1 mRNA and protein are present in high density in the trigeminal ganglion (TG) and in the sensory axons of the tooth pulp (7–9). In addition, cultured human odontoblast-like cells (10) and cultured dental pulp fibroblasts (11) exhibit cold-induced increases in intracellular calcium *in vitro*, which is partly explained by their TRPA1 and TRPM8 channels. However, acutely isolated native human odontoblasts express TRPM8 but not TRPA1 (12), in contrast to these cells in more prolonged culture (10, 13). In rat odontoblasts *in vitro*, cold sensitivity is controversial (14, 15). The physiological significance of the observed cold transduction in odontoblast and fibroblasts is still unclear, because the specific contributions of TRPA1 and TRPM8 to cold-induced tooth pain *in vivo* were not observed (16) and electrophysiological models to directly examine the relevance of these channels in the tooth sensory system are missing. Thus, the cold transduction molecules and site of transduction in teeth remain

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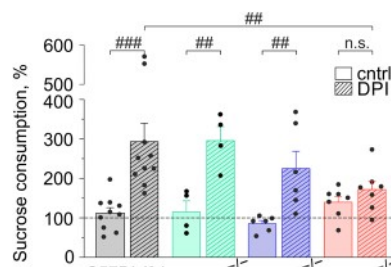
RESULTS

TRPC5 is required for inflammatory tooth pain

To evaluate cold transduction ion channels for inflammatory tooth pain, we first used an established dental pulp injury (DPI) model (18) in TRPC5, TRPA1, and TRPM8 null mice. A major sign of painful DPI in mice is a paradoxical increase in sucrose consumption (18). Administration of the anti-inflammatory analgesic indomethacin is sufficient to reverse the levels of sucrose consumption to that of uninjured controls (18). We found that DPI enhances the consumption of 5% room temperature sucrose water to ~3-fold above baseline (Fig. 1B). In TRPA1^{-/-} and TRPM8^{-/-} mice, DPI induced a comparable increase in sucrose consumption. Notably, only in TRPC5^{-/-} mice was sucrose consumption reversed to the level of the uninjured controls (Fig. 1B), similar to the effect of indomethacin (18). These data indicate that cold-sensing TRPC5 is relevant and necessary for inflammatory tooth pain-associated behavior.

To enable functional examination of the entire tooth sensory system, we developed an intact mouse mandible-inferior alveolar nerve (jaw-nerve) preparation (Fig. 1B). The *ex vivo* jaw-nerve preparation enables the recording of propagated action potentials from the inferior alveolar nerve to sensory stimuli in the mandibular incisor and molars, similar to recordings from nociceptors of the saphenous nerve with receptive fields in the skin (6). In contrast to the skin-nerve model in which recordings are made from finely split nerves (19), the inferior alveolar nerve in the jaw-nerve model is too short to enable these recordings. Therefore, we used a suction electrode to record voltage changes directly from the intact nerve end (Figs. 1B and 3A).

In this preparation, we focused on cold stimulation because it is adequate to produce tooth pain and activates the TRPC5 current in heterologous expression (17). When the intact jaw was exposed to cold, we observed large responses from ~10% of A- and C-fibers. Tooth cold receptor neurons fired 114 ± 103 action potentials per cold stimulus, with peak firing frequencies of 43 ± 10 per second. These values far exceed mouse skin nociceptor cold responses (16 ± 8 action potentials and firing frequencies of 3 ± 1 per second; Fig. 1B and C). The thresholds of the tooth pulp nociceptors were 19 ± 1°C, 2°C lower than the cold nociceptors in the skin (cutaneous nociceptor threshold 21 ± 1°C; Fig. 3D). There were no notable differences in cold responses between A- and C-fibers in these specific parameters (fig. S2).



TRPC5 is a cold sensor in intact teeth

In the jaw-nerve preparation, the TRPC5 blockers HC-070 and ML204 effectively eliminated some tooth nociceptor's cold responses and, on average, reduced cold responses by 59 ± 16% (Fig. 4A and B). ML204, which also blocks TRPC4 and TRPC3 channels, had no effect in TRPC5^{-/-} mice, suggesting that TRPC3 and TRPC4 were not involved as homomeric channels in cold responses. In addition, the TRPC5 agonist riluzole sensitized the cold response in TRPM8^{-/-} jaws (fig. S3). In all fibers that remained unblocked by HC-070, the TRPA1 blocker HC-030031 abolished the remaining cold responses (97 ± 5%; Fig. 4A and B). Furthermore, in the jaw-nerve preparations from TRPC5^{-/-} mice, we found that the number of cold nociceptors was reduced by roughly half (Fig. 4C). We noted that although the remaining TRPC5^{-/-} tooth nociceptors had unchanged response magnitudes, they had higher peak firing rates than any other strain (116 ± 34 action potentials, discharge rate of 66 ± 10 per second) and were activated at high threshold temperatures

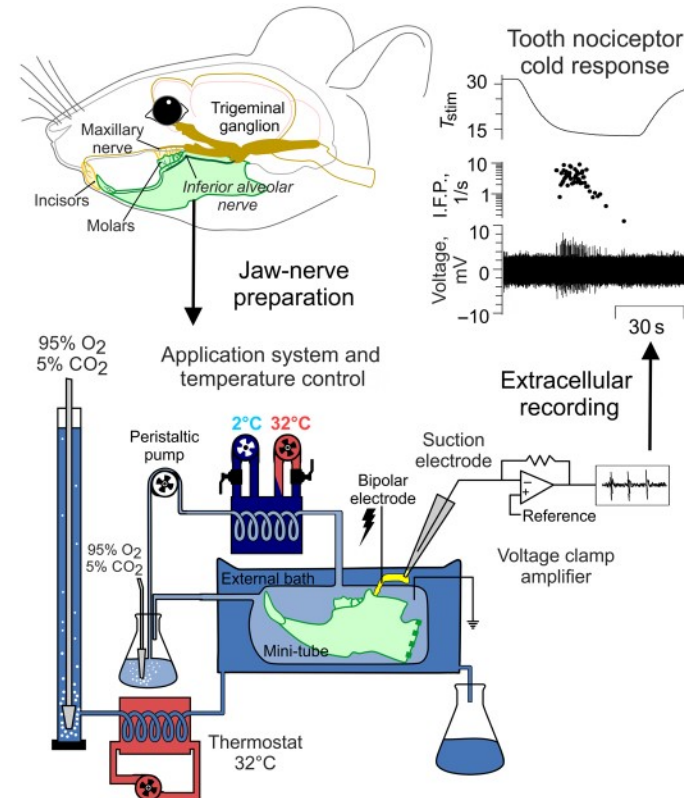


Fig. 2. Extracellular recordings from mouse tooth nociceptors in a novel jaw-nerve preparation. (Top) Illustration of mouse head with jaws and their innervation. The mandible-inferior alveolar nerve preparation is derived from the lower jaw and transferred to an organ bath consisting of an external solution and a mini-tube that is connected to a temperature-controlled application system. Con-

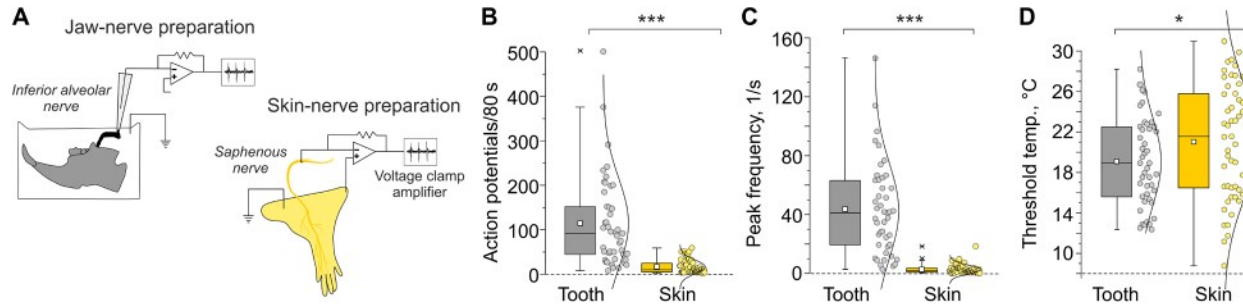


Fig. 3. Tooth nociceptor cold responses are much larger than skin cold nociceptor responses. (A) Schematic illustration of extracellular recordings from jaw-nerve as compared to skin-nerve preparations. (B to D) Comparison of the (B) cold response magnitude, (C) peak frequency, and (D) threshold temperature of C57BL/6J teeth ($n = 45$) and skin ($n = 59$) nociceptors. Statistical significance was identified by a two-sided Student's *t* test: *** $P = 2 \times 10^{-8}$, *** $P = 3 \times 10^{-11}$, and * $P = 0.05$. Skin nociceptor cold responses are from (6, 50) and refer to the same background strain at equivalent stimulus conditions.

(22.50°C; Fig. 4B and C). Although in mouse skin most of the cold responses are TRPM8-mediated (6), the teeth cold responses appeared insensitive to pharmacological TRPM8 modulation (fig. S4).

The deficits in the prevalence of TRPC5^{-/-} cold responses, combined with the high efficacy of HC-070 and HC-030031 blockers, suggest that TRPC5 and TRPA1 are essential for cold sensing in teeth. In jaw-nerve preparations derived from TRPC5/A1-DKO (double-knockout) mice, we found that the number of cold responses was reduced by one-third (to <3%; Fig. 4C). Moreover, these cold responses had reduced response magnitudes and action potential firing rates compared to normal tooth nociceptors (20.50 action potentials, discharge rate of 9.50 per second) and were activated at very low temperatures (15.50°C; Fig. 4D and E). These small responses were insensitive to fast temperature drops (Fig. 4B and F), and TRPM8 blockers or agonists had no effect (Fig. 4D).

We conclude that TRPC5 and TRPA1 are sufficient for cold transduction in healthy teeth. These findings raise the question of whether the specific anatomical context in teeth is required for TRPC5 cold sensitivity or if TRPC5 is also cold sensitive in the isolated cell bodies derived from the primary afferent terminals.

Dental primary afferent neurons (DPANs), the cell bodies of the sensory terminals in the maxillary plexus of Raschkow, are clustered in the cranial TG and were identified by retrograde labeling (7). Dissociated and in culture, they represent a model for the transduction processes in the tooth pulp's sensory nerves (fig. S5). We screened the red retro-labeled DPANs for cold-induced changes in [Ca²⁺] and chemical responsiveness to the TRPM8 agonist menthol, the TRPA1 agonist carvacrol, the TRPC5 agonist riluzole, and the antagonist ML204. We identified ~17% of neurons as cold sensitive. Of these, 74% were sensitive to menthol and 57% to carvacrol, but only one neuron (<1%) was sensitized by the TRPC5 agonist riluzole (Fig. 5A and B). Because menthol and carvacrol are not highly specific and can activate both TRPM8 and TRPA1, we also used DPANs derived from DKO strains, lacking TRPC5 and TRPM8 (TRPC5/M8-DKO), TRPC5^{-/-}TRPA1 (TRPC5/A1-DKO), or

observations match previous observations from dorsal root ganglion neurons (20).

Similar to DPANs derived from TRPC5/M8-DKO mice, DPANs from TRPM8/A1-DKO mice had few cold-sensitive cells (~5%) and none of these cells were activated by carvacrol or menthol (Fig. 5A). The TRPC5 blocker ML204 reduced their remaining cold-induced activity by 90% (Fig. 5B and C). ML204's blocking effect was also found in the background strain (23% reduction), but absent in TRPC5^{-/-} DPANs, where virtually all cold-sensitive DPANs were sensitive to menthol (93%; Fig. 5A and B). In examining the cold-activated [Ca²⁺] kinetics, TRPM8/A1-DKO DPAN signals were smaller and rose more slowly compared with the fast-rising response of menthol-sensitive neurons (fig. S6). To test whether total activity (approximated by the slope of the cold-activated [Ca²⁺] signals) of the TRPM8/A1-DKO DPANs and riluzole-induced sensitization of the cold response represented functional TRPC5, we compared it to the responses of mTrpc5 in human embryonic kidney (HEK) 293T cells and found both response patterns to be similar and riluzole to largely increase TRPC5 cold responses (fig. S6). In summary, nerve (DPAN) cold responses are dominated by TRPM8 with smaller and slower contributions by TRPC5 and TRPA1 channels.

TRPC5 cold transduction originates in odontoblasts

Consistent with the small number of nerve cells with functional TRPC5, a transcriptomic analysis from acutely dissociated labeled DPANs in the background strain identified *Trpm8* and *Trpa1*, while *Trpc5* was below the detection threshold (Fig. 6A and table S1). However, we visualized and quantified TRPC5 protein by multiphoton microscopy in retrograde-labeled whole TGs of reporter mice expressing tau-GFP (green fluorescent protein) under the control of the TRPC5 promoter (21). We focused on areas from the mandibular and maxillary branches that have the largest density of DPANs (7) and identified 3.5% TRPC5⁺ DPANs (Fig. 6B), similar to the 2.2 to 4.8% cold-sensitive, carvacrol- and menthol-insensitive DPANs in the background strain C57BL/6J. TRPM8/A1-DKO

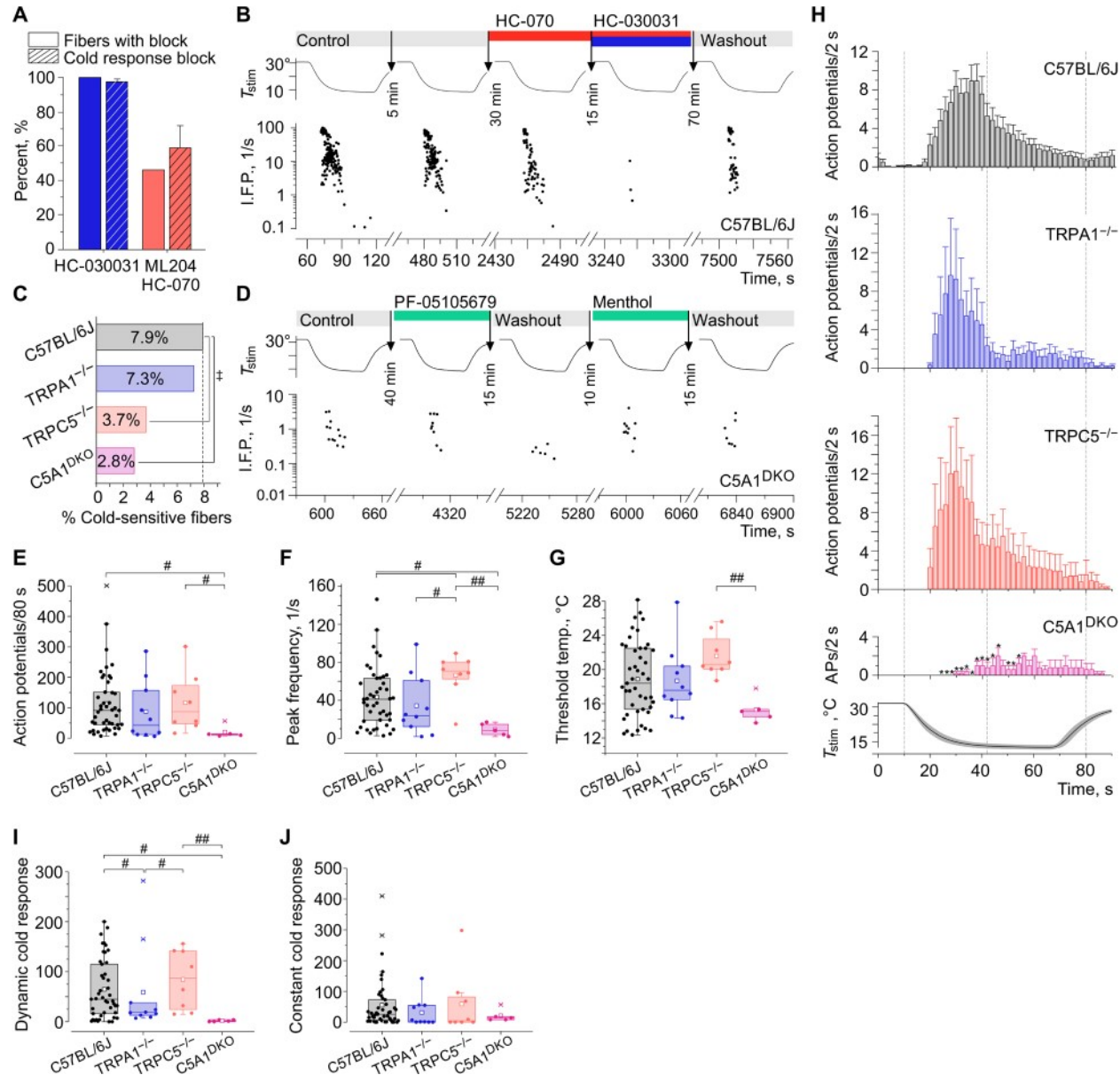


Fig. 4. TRPC5 and TRPA1 are sufficient as cold sensors in healthy teeth. (A) Percent cold-sensitive tooth nociceptors blocked by HC-030031 ($n = 9$ of 9 fibers) and ML204/HC-070 ($n = 6$ of 13 fibers) and respective fraction of block (means \pm SEM). (B) C57BL/6J wt tooth nociceptor recording with temperature (top) and instantaneous frequency pattern (I.F.P.; bottom) blocked by HC-070 and HC-030031. Circles represent action potentials. (C) Tooth cold responses in TRPA1^{-/-} ($n = 10$ of 138, $P = 1.0$), TRPC5^{-/-} ($n = 8$ of 217, $^{\dagger}P = 0.04$), and TRPC5/A1-DKO ($n = 5$ of 177, $^{\dagger}P = 0.02$), chi-square tests versus C57BL/6J ($n = 45$ of 570). (D) Typical cold response of a TRPC5/A1-DKO tooth nociceptor with temperature (top) and instantaneous frequency pattern (I.F.P.; bottom). Action (B) circles represent action potentials and horizontal bars and arrows

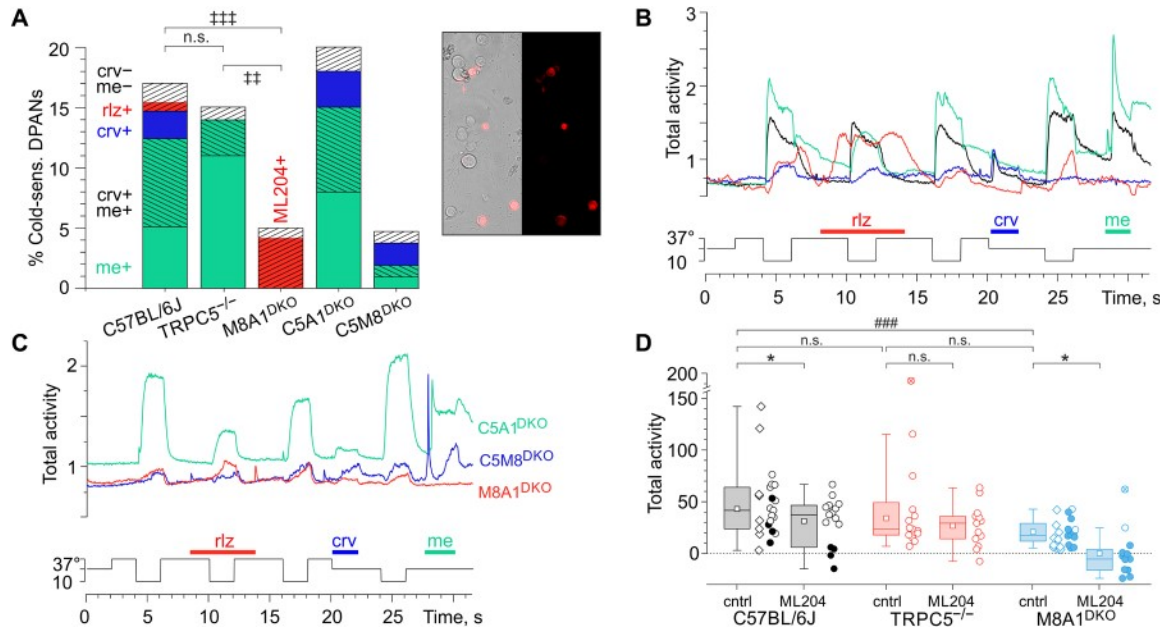


Fig. 5. Most cold-sensitive DPANs use TRPM8, but TRPA1 and TRPC5 are also functional cold transducers. (A) Characterization and quantification of cold-sensitive cultured DPANs (C57BL/6J; $n = 23$ of 136) based on Ca^{2+} transients measured with Fura-2 AM and sensitivity to TRP channel modulators menthol (me, $n = 7$), carvacrol (crv, $n = 3$), both ($n = 10$, descending diagonal stripes), neither ($n = 4$, ascending diagonal stripes), and riluzole (rlz, $n = 1$). Cold-sensitive DPANs insensitive to menthol and carvacrol (ascending diagonal stripes) are unchanged in $TRPC5^{-/-}$ ($n = 1$ of 14 in 93) versus C57BL/6J (3 of 23 in 136; n.s. $P = 0.86$), but increased in $TRPM8A1$ -DKO ($n = 23$ in 457; $\#P = 0.002$ and $\#\#\#P = 0.00003$). In $TRPC5A1$ -DKO, cold-sensitive cells ($n = 20$ of 100) were mostly menthol sensitive ($n = 15$; crv+ $n = 10$; both, $n = 7$ and none, $n = 2$). $TRPC5M8$ -DKO cold-sensitive cells ($n = 5$ of 103) were mostly sensitive to carvacrol ($n = 3$; me+ $n = 2$; both, $n = 1$ and none, $n = 1$). Inset: Photomicrograph of cultured mouse TG neurons with red Dil retro-labeled DPANs. (B and C) Ca^{2+} transient traces from (B) C57BL/6J representative of four types of cold-sensitive neurons and (C) $TRPM8A1$ -DKO (red), $TRPC5A1$ -DKO (green), and $TRPC5M8$ -DKO (blue). Bottom: Temperature stimulator command. (D) $TRPM8/A1$ -DKO control (cntrl) cold responses ($n = 23$) were smaller than in C57BL/6J ($n = 23$; $\#\#\#P = 0.0007$) but not in $TRPC5^{-/-}$ neurons ($n = 13$; $P = 0.2$). ML204 (circles = treated neurons) blocked most cold responses in $TRPM8/A1$ -DKO ($\geq 50\%$ block = filled circles, $n = 12$; $*P = 0.01$) and some in C57BL/6J neurons ($n = 15$; $*P = 0.02$), but not in $TRPC5^{-/-}$ neurons ($n = 13$; n.s. $P = 0.2$). n.s., not significant. Lines are medians, squares mean, boxes IQR, whiskers 2.2-fold IQR after exclusion of >2.2 IQR outliers identified by crosses.

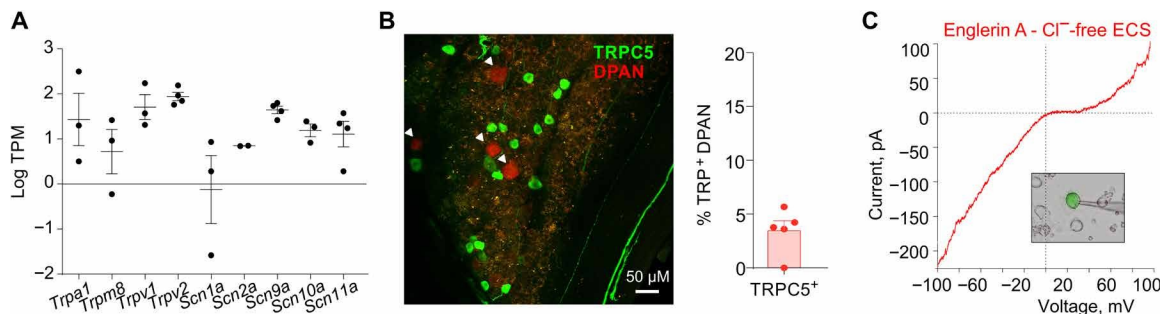


Fig. 6. Functional TRPC5 in trigeminal neurons. (A) Relative expression of TRP channel genes and nociceptor-specific subtypes of voltage-gated sodium channel genes in mouse DPANs given as transcripts per million (TPM). Data are presented as means \pm SEM. Dots represent each replicate. Replicates with value 0 are not represented in the logarithmic scale. TRPC5 was not among the transcripts (see table S1). (B) Red DPANs (arrowheads) in $TRPC5$ reporter mouse ganglion (TG) multiphoton stacks of maxillo-mandibular regions. A total of 176 DPANs had 11 $TRPC5^{+}$ neurons (seven TGs, five mice). (C) Typical doubly rectifying current-voltage relation observed in a cultured $TRPC5^{+}$ neuron of a re-

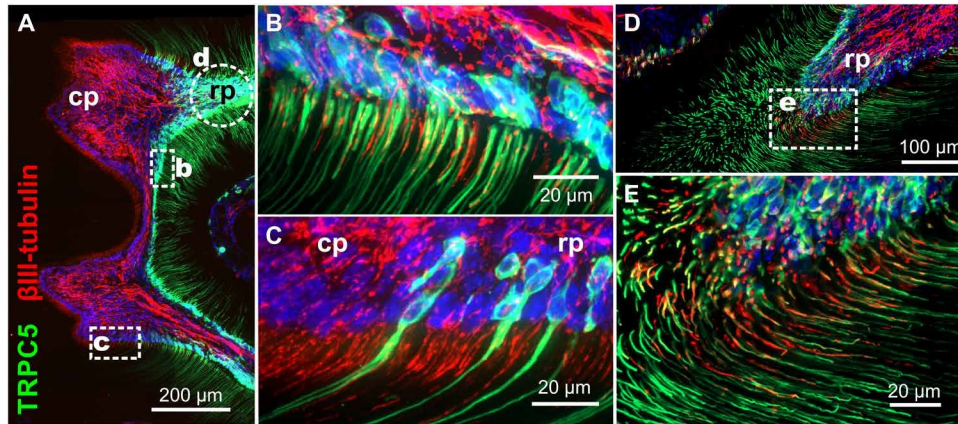


Fig. 7. TRPC5 channels are located in the odontoblast layer. (A) TRPC5 reporter mouse molar tooth whole mount with densely packed TRPC5⁺ odontoblasts at the pulp-dentin boundary. (B and C) In tight association with sensory nerves. Green, TRPC5; red, β III-tubulin; circle indicates area shown in (D) and (E) from a subsequent section. cp, coronal pulp; rp, radicular pulp. (E) Oblique section through the predentinal radicular pulp to visualize the tight association of TRPC5⁺ odontoblast processes with their sensory nerves.

alveolar nerves and radiating through the root into the nerve plexus of Raschkow or in the inferior alveolar nerve (Fig. 7 and movies S1 and S2). Thus, TRPC5, in contrast to the ubiquity of TRPM8 and TRPA1 in anatomical compartments of mouse and human teeth (fig. S7) (7, 9, 13, 50), is largely restricted to the odontoblast cell layer.

Ideally, TRPC5 should also be recorded in response to cold in odontoblasts in the intact tooth system of live, unanesthetized mice, but this has not yet been technically possible. Isolated odontoblasts do not fulfill this need because gene expression may be altered during isolation and culture. In addition, the respective functional phenotype depends on the origin of the cells, methods used for the induction of odontoblastic differentiation (12), developmental stage, intradental location, and innervation of the odontoblasts (23). A recent study, based on TRP channel expression patterns, implied that rat odontoblasts do not acquire potential sensory function before they begin to form root dentin (24). With these caveats in mind, we propose that TRPC5 expression and cold sensing indicate an essential sensory receptor function for the odontoblasts in the transduction hierarchy from the enamel to the dentinal primary afferent nerves.

TRPC5 is increased in human teeth with pulpitis

A remaining question is whether TRPC5 is also a cold sensor in human teeth and expressed in human odontoblasts and how it is affected in caries and inflammatory conditions associated with injured or patent pulp. In healthy human teeth from adults, removed for orthodontic or cosmetic reasons, we identified TRPC5⁺ in the odontoblastic layer. We found more TRPC5 than TRPM8⁺ sensory nerves at the pulp-dentin boundary and in dentinal tubules. The sensors (25) at the pulpo-dentinal border zone (type II/III) exhibited the largest proportion of TRPC5 colabeling. In addition, we found a

significantly higher percentage of TRPC5⁺ pulp and root fibers (Fig. 8, F and G and fig. S9). We also observed TRPC5⁺ type IV fibers in the degenerating dentin (Fig. 8H). The higher percentage of TRPC5⁺ in pulpitic teeth and the presence of dentinal fibers within the normal and degenerating dentinal tubules suggest that TRPC5 also acts as a cold sensor in human teeth.

DISCUSSION

Dentine sensitivity as a thermomechanical sensation

This study aimed to find the molecular cold-sensing mechanism in teeth. Using a novel jaw-nerve preparation that enabled us to record propagated electrical activity from intact teeth, we identified TRPC5 and TRPA1 as the molecular cold sensors and odontoblasts as the site of cold transduction. This experimental model allowed us to close an important gap in our understanding of tooth pain as it is the first model that allows the assessment of the tooth sensory system in its entire anatomical and, necessarily, physiological context in transgenic mice. This is critical because, unlike anywhere else in the body, the dental functional anatomy poses complex problems to sensing damaging physical stimuli. The odontoblast cellular layer's location in the outermost zone of the tooth pulp makes it a natural barrier between the mineralized hard tissues and the soft dental pulp. Each odontoblast has a process protruding in a dentinal tubule where it is immersed in dentinal fluid. Its cell body and process are surrounded by unmyelinated sensory nerves within the first 100 μ m of the odontoblast-predentin border (fig. S1) (26). This unique hierarchical arrangement, from macroscale enamel via fluid-filled dentinal microtubules to molecular level ion channels, provides the structural basis for the encoding of thermal pain (3).

