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Variability in Capsaicin-Stimulated CGRP Release from Human Dental Pulp

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Abstract

Introduction—The unique innervation and anatomical features of the dental pulp contribute to the remarkable finding that any physical stimulation of pulpal tissue is painful. Further, when pathological processes, such as caries, affect teeth, and produce inflammation of the pulp, the pain experienced can be quite intense and debilitating. To better understand these underlying neurobiological mechanisms, and identify novel analgesic targets for pulpally derived pain, we have developed a powerful *ex vivo* model using human tooth slices.

Methods—Non-carious, freshly-extracted teeth were collected and sectioned longitudinally into 1mm thick slices containing both dental pulp and the surrounding mineralized tissues. Tooth slices from 36 patients were exposed to 60 uM capsaicin to stimulate the release of calcitonin gene-related peptide (CGRP) from nerve terminals in the pulp. Patient factors were analyzed for their effects on capsaicin-stimulated CGRP release using a mixed model ANOVA.

Results—Approximately 1/3 of the variability observed in capsaicin-evoked CGRP release was attributable to differences between individuals. In terms of individual factors, there was no effect of anesthesia type, sex or age on capsaicin-stimulated CGRP release. Using a within-subject study design, a significant effect of capsaicin on CGRP release was observed.

Conclusions—Capsaicin-stimulated CGRP release from dental pulp is highly variable between individuals. A within-subject study design improves the variability and maximizes the potential of this powerful translational model to test the efficacy of novel pharmacotherapeutic agents on human peripheral nociceptors.

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Introduction

The dense network of sensory neurons that innervate dental pulp have unique anatomical and neurochemical characteristics, such that understanding how they transduce pain is of interest to both clinicians who strive to minimize the experience of dental pain for their patients, and to scientists seeking to understand how the peripheral nervous system detects pain and communicates that signal to the brain (1). When dental pulp tissues become inflamed, a patient can experience intense pain that disrupts the basic activities of daily living including eating, sleeping and participating in work or school (2,3). Although initiating root canal treatment is usually an effective way to treat painful pulpitis, novel pain management strategies are needed to control pain pre- and postoperatively, and, in certain situations, to allow the possibility to control pain after caries removal, while preserving the pulp. Translational models using human pulp allow for the opportunity to study the function of pulpal afferents and identify novel analgesic strategies.

Symptomatic pulpitis is characterized by cold, heat, and mechanical hypersensitivity, as well as spontaneous pain (4). Members of the transient receptor potential (TRP) family are involved in both the detection and transduction of thermal, mechanical, and chemical stimuli, and are important molecular sites for integration of inflammatory signaling pathways (5,6). Several TRP receptors, including TRPV1, are found in various cell types of the pulp, including sensory afferents and odontoblasts (7–11). TRPV1 responds to noxious heat and the selective agonist, capsaicin. The activation of TRPV1 in neurons can stimulate the release of neurotransmitters, including calcitonin gene-related neuropeptide (CGRP), a pro-inflammatory vasodilator, which contributes to peripheral and central sensitization in the trigeminal sensory system (12–16). The use of capsaicin-stimulated CGRP release as a primary endpoint is a well-established model for evaluating peripheral afferent activation, and the ability to use human tissue, as described in this study, provides a powerful translational model to test the effects of novel pharmacological compounds on human pulpal nociceptor activity (17–20). In this study we characterized a model in which capsaicin-stimulated CGRP release was measured from dental pulp within human tooth slices. Further we explored whether subject-specific predictors influenced these measures. Understanding the contribution of patient factors to the variability observed in stimulated neuropeptide release broadens our understanding of the utility of this translational model, as well as provides insight into the most effective experimental design for future studies.

Materials and Methods

Patient Recruitment/Sample Collection

Molar teeth were collected from patients at the New York University College of Dentistry Oral Surgery Clinic. Inclusion criteria for this study were molars having fully-formed roots, no visible caries penetrating into dentin, and no existing restorations. Teeth that were fractured or sectioned during extraction were excluded. Prior to extraction, all patients were anesthetized using local anesthetic (2% lidocaine with 1:100,000 epinephrine), with some patients also receiving intravenous (I.V.) sedation (propofol). The subjects' age and sex were recorded. Freshly extracted teeth were placed in ice-cold phosphate-buffered saline (PBS) and kept on ice until ready for processing. The amount of time elapsed between extraction

and pulp stimulation ranged from one to five hours. In total, 58 teeth were collected from 36 patients.

Tissue Processing

After removing attached periodontal tissues, teeth were rinsed and disinfected by submersion in fresh PBS, 70% ethanol, and 4% sodium hypochlorite solution. Disinfected teeth were mounted to a sectioning block via impression compound, then attached to an IsoMet low-speed saw (Buehler; Lake Bluff, IL) for longitudinal sectioning. Impression compound was briefly heated to make it pliable and cooled before mounting each tooth. The first cut with the saw was made to expose the pulp chamber, followed by a subsequent cut that served to create a tooth slice comprised of hard and soft tissue, with a thickness of approximately 1mm. Sectioning was repeated to obtain multiple slices (usually 2–3 per tooth) containing pulp. Tooth slices were visually examined for the presence of vital pulp tissue, and any teeth with necrotic or partially necrotic pulps were excluded from the study.

CGRP Release Assay

Tooth slices were stored in cold PBS until sectioning. For the release assay, slices were placed into wells containing Hank's balanced salt solution buffer (HBSS) (15.5 mM dextrose, 10 mM HEPES, 4.0 mM NaHCO₃, 0.1% BSA, pH 7.4, in HBSS, Life Technologies/Gibco; Carlsbad, CA) for a 15 minute equilibration period. All subsequent steps were completed at 37°C in a humidified incubator with 5%CO₂/95%O₂. Slices were then immersed in HBSS buffer containing 60 uM capsaicin (Sigma, St. Louis, MO) or vehicle (HBSS buffer with capsaicin vehicle only) for 30 minutes to stimulate CGRP release. To determine total CGRP content after the release assay, pulp tissues were lysed by heating to 90°C for ten minutes followed by cooling on ice for five minutes.

ELISA

CGRP concentration was determined using a human CGRP-specific enzyme immunoassay (SPI-BIO, Montigny le Bretonneux, France). Samples were thawed and concentrated using vacuum centrifugation to a final volume of 200uL. The ELISA protocol was completed as recommended by the manufacturer. Plates were read using a SpectraMax M5 microplate reader with SoftMax Pro analysis software (Molecular Devices, Sunnyvale, CA). A standard curve was included in each plate, as well as an internal quality control sample, both supplied by the manufacturer as part of the kit.

Statistical Analysis

Data are presented as the percentage of total content of CGRP released per slice. Total content of CGRP was calculated as the stimulated release amount plus the amount released by the lysis protocol. Preliminary inspection of the data showed that this measure was distributed in a reasonably symmetric manner. A mixed model ANOVA was used to compare the percentage of CGRP total content while modeling a random subject intercept as a function of age, sex, type of anesthesia received, dental arch (maxilla or mandible), and time between tooth extraction and experimentation. A subset of samples were used for the within-subject analysis, in which slices from the same subject were treated with either

vehicle or 60 μ M capsaicin. Those treatments were compared in a mixed model ANOVA with fixed factors of condition and a random intercept. Because the analytic approach was determined after the data was collected, and data did not exist data to base it on, a power analysis was not completed. Finally, p -values less than 0.05 were considered statistically significant.

RESULTS

A total of 58 molar teeth were collected from 36 patients between the ages of 13 and 68 years old. From these teeth, 117 slices were obtained for experimentation. Table 1 shows the number of teeth and slices obtained for each variable classification (sex, anesthesia type, and dental arch).

A wide variability in capsaicin-stimulated CGRP release was observed among subjects, ranging from none to as much as 60% of total content (Figure 1). On average, about 25% of total CGRP content is released upon stimulation with 60 μ M capsaicin. [Mean(Standard Deviation)= 27.9% (14.2)]. We observed several outliers in the distributions of both the total content of CGRP [range= 1.8–286.4 pg/ml; M(SD)= 47.4 (40.7); data not shown] and raw (uncorrected) measures of capsaicin-stimulated CGRP release [range= 0–84.3 pg/ml; M(SD)= 13.4 (12.9); data not shown]. Importantly, when the data were normalized as the percent-release of total content, there were fewer outliers and less variability in the distribution [range 0–79.0 %; M(SD)= 27.9 (14.1)]. These observations support the use of the percent-release of total content as an appropriate way to normalize the data, and all subsequent analyses utilize this method of normalization.

We investigated five subject variables for their effects on capsaicin-stimulated CGRP release (Fig 2): sex, anesthesia type (IV sedation and local anesthesia versus local anesthesia only), maxillary versus mandibular arch, age, and the latency between extraction and experiment start. None of these factors are related to capsaicin-stimulated CGRP release [sex (Fig 2a; $p=0.52$); anesthesia type (Fig 2b; $p=0.98$); arch (Fig 2c; $p=0.51$); age ($r=0.12$, $p=0.40$, data not shown) time from extraction to initiating assay ($r=-0.01$, $p=0.54$, data not shown).

Results from a subset of slices ($n=11$ per group) were analyzed using a within-subject approach to compare 60 μ M capsaicin- to vehicle-stimulated CGRP release; i.e. only results from slices obtained from the same patient were compared (Fig 3). Slices exposed to capsaicin released more CGRP [M(SD)= 22.8% (14.1)] than those exposed to vehicle [M(SD)= 7.9% (8.3)]. Analysis also revealed that about 1/3 of the within-cell variance was attributable to consistent differences between subjects (i.e., of 151.1 units of total residual variance, 56.1 were attributable to the intercept). The observed variability between subjects suggests that a within-subject design is a more efficient way of evaluating neuropeptide release in this model.

DISCUSSION

In this study, we investigated whether patient variables (age, sex, type of anesthesia received, and dental arch), as well as latency between tooth extraction and experimentation, affected the magnitude of capsaicin-stimulated CGRP release from dental pulp. In agreement with a

previous study we found no significant effect for any of these individual factors (21). This finding is important because it suggests that excluding subjects meeting these specific criteria would be unlikely to reduce the observed variability in the primary outcome, i.e. excluding females, or patients who received i.v. sedation. However, despite the lack of significant individual factors, we did observe significant variation between subjects in capsaicin-stimulated CGRP release, which overall accounted for about 1/3 of the variance in the results. So although the patient-specific factors we analyzed did not independently influence the variability, there is still a significant amount of variability in capsaicin-stimulated CGRP release between individuals. We also identified that normalizing data to the percent of total CGRP content of the slice improved the variability in the data.

This study utilizes activation of the TRPV1 receptor by capsaicin to evoke CGRP release from human dental pulp as a model for studying peripheral nociceptor pharmacology. Previous studies using human dental pulp from extracted teeth have completely dissociated the dental pulp from the surrounding hard tissues (21,22). In this study, the use of *ex vivo* tooth slices maintains the gross anatomical relationships present in the pulpo-dentin complex, specifically the orientation of the nerve endings to dentinal tubules and odontoblasts. Preservation of this physiologic architecture may prove to be essential in studying the sensory capacity of the dental pulp. Indeed, recent findings describing functional TRP receptors, sodium channels, and purinergic receptors in odontoblasts and other cells in dental pulp, suggest that we should not overlook the important role of non-neuronal cells in nociception (9,23). Whether odontoblasts can sensitize or activate sensory afferent neurons remains unclear, but the close physical relationship of the two cell types at the pulp-dentin interface allows for the possibility of paracrine signaling (24).

Between-subject heterogeneity is a well-recognized complicating factor in clinical/translational studies involving somatosensory testing and pharmacologic studies. For example, studies using microdialysis to evaluate pharmacokinetics of dermal drug application reported between-subject variability to be the primary source of variability in the assay, complicating the measurement of drug bioequivalence (25,26). Human experimental pain models using capsaicin or heat to evoke secondary hyperalgesia also find that between-subject heterogeneity is a major source of variability (27). Inherent individual differences in pain neurotransmission create a challenge in the design of clinical/translational pain studies using human subjects or tissues. Our studies further confirm the presence of significant heterogeneity between individuals in the basic functioning of neurotransmitter release from nociceptive nerve endings in dental pulp.

The potential factors contributing to the heterogeneity observed between individuals in their capsaicin-stimulated CGRP from pulp are numerous, but, broadly speaking, this variability points to the inherent diversity in nociceptive responses observed across individuals. Studies in humans and rodents have identified that variability in the expression of genes predicts an individual's response to specific physiologic stimuli, and susceptibility to chronic pain (28,29). As such, differential expression of the genes encoding TRPV1 and/or CGRP could account for some of the individual variability in capsaicin-stimulated CGRP release. In mice, differential expression of TRPV1 and CGRP contribute to between-strain differences in thermal sensitivity (30,31). In humans, CGRP, substance P, and neurokinin A levels are

significantly higher in pulps from symptomatic teeth versus those from healthy teeth (32). Thus, although we did not test this in our experiments, perhaps an individual who released larger amounts of CGRP in response to capsaicin would have a lower pain threshold in response to heat stimulation, or a more painful response to deep caries. Clearly many intriguing and critical questions remain in this area of research.

We did not see a difference between slices originating from males and females in capsaicin-stimulated CGRP release, despite the fact that gender is a critical influencer of an individuals' pain experience (33). Our findings may suggest that gender based differences in pain processing may primarily involve the central nervous system, and may not manifest at the level of the primary afferent, at least as measured in the present study. A similar inference may be drawn from our finding that anesthesia type did not significantly affect capsaicin-induced CGRP release, consistent with the action of general anesthetics on the CNS. However, we cannot rule out that our experimental design prevents us from observing influences that are important modulators *in vivo*.

Our results provide a foundation for the design and implementation of future experiments. Our analyses show that using a within-subject experimental design (Fig 3 data) versus a between-subject design (Fig 2 data) reduced the minimal detectable effect size by about 20%. That is, when using a within-subject design to test a hypothesis (e.g. does drug 'x' produce a significant increase in CGRP release compared to drug 'y' and/or vehicle?), we are able to detect differences between treatment groups that would be 20% smaller than if we were using a between-subject design. This provides a strong rationale for using within-subject designs in future studies.

Determining the predictive validity of novel pharmaco-therapeutic targets identified using animal models and translating pre-clinical hypotheses into therapies are some of the greatest challenges the field of pain research faces (34). In this study, we use a human model circumventing this limitation. By obtaining multiple tooth slices from an individual, a within-subject experimental designs can be implemented, whereby each subject acts as his/her own control, thereby reducing the influence of subject-to-subject variability. This results in more powerful studies requiring fewer subjects. A better understanding of the mechanisms contributing to variance in neuropeptide release from human dental pulp will help to further validate and discover the limitations of the human tooth slice model.

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Figure 1.

Heterogeneity in capsaicin-induced CGRP release from human tooth slices. Each data point on this figure shows the mean amount of CGRP released in response to 60 μ M capsaicin from individual tooth slices obtained from each subject. Some subjects only contributed a single slice, so only a single data point is shown. Release is presented as the % of total CGRP content, mean $\pm$ SEM.

Figure 2.

Effect of patient factors on capsaicin-induced CGRP release from human tooth slices. The analyzed patient factors did not influence the amount of capsaicin-stimulated CGRP release from tooth slices. (A) Sex. 42 teeth were collected from 23 female patients, yielding 88 tooth slices. 16 teeth were collected from 13 male patients, yielding 29 tooth slices. (B) Anesthesia type. 26 teeth were collected from 10 patients receiving I.V. anesthesia, yielding a total of 59 tooth slices. 32 teeth were collected from 26 patients receiving only local anesthetic, yielding 58 tooth slices. (C) Dental arch. 35 maxillary molars were collected, yielding 69 tooth slices. 23 mandibular molars were collected, yielding 48 tooth slices.

Figure 3.

Evaluation of CGRP release using a within-subject experimental design. Release was measured for 11 subjects. Difference in amount of CGRP released from subject matched tooth slices treated with either capsaicin (60 μ M) (CAP) or vehicle (VEH). CGRP levels were normalized to total CGRP content. The slices treated with capsaicin released more CGRP than those treated with vehicle (paired two-tailed t-test, $p < 0.01$, $n = 11$).

Table 1

Numbers of teeth and slices obtained by variable/patient factor

Total Subjects	36			
Total Teeth	58			
Total Sections	117			
Subject Variables				
Gender	Male: 13 (36%)		Female: 23 (64%)	
	Teeth	Sections	Teeth	Sections
	16	29	42	88
Anesthesia Type	I.V.: 10 (28%)		Local only: 26 (72%)	
	Teeth	Sections	Teeth	Sections
	26	59	32	58
Arch	Maxillary		Mandibular	
	Teeth	Sections	Teeth	Sections
	35	69	23	48
Mean Age (standard deviation)	30.9 (14.9)			
Mean time in minutes from extraction until stimulation (standard deviation)	177.1 (75.8)			