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Research paper

Multiple complex somatosensory systems in mature rat molars defined by immunohistochemistry

Margaret R. Byers*, Leanne M. Cornel

Department of Anesthesiology & Pain Medicine, Box 356540, University of Washington, Seattle, WA 98195-6540, USA

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ABSTRACT

Objective: Intradental sensory receptors trigger painful sensations and unperceived mechanosensitivity, but the receptor bases for those functions are only partly defined. We present new evidence here concerning complex endings of myelinated axons in rat molars.

Design: We sectioned mature rat jaws in sagittal and transverse planes to analyze neural immunoreactivity (IR) for parvalbumin, peripherin, neurofilament protein, neurotrophin receptors, synaptophysin, calcitonin gene-related peptide (CGRP), or mas-related g-protein-receptor-d (Mrgprd).

Results: We found two complex sensory systems in mature rat molar dentin that labeled with neurofilament protein-IR, plus either parvalbumin-IR *or* peripherin-IR. The parvalbumin-IR system made extensively branched, beaded endings focused into dentin throughout each pulp horn. The peripherin-IR system primarily made unbeaded, fork-shaped dentinal endings scattered throughout crown including cervical regions. Both of these systems differed from neuropeptide CGRP-IR. In molar pulp we found peripherin- and parvalbumin-IR layered endings, either near special horizontal plexus arrays or in small coiled endings near tangled plexus, each with specific foci for specific pulp horns. Parvalbumin-IR nerve fibers had Aβ axons (5–7 μm diameter), while peripherin-IR axons were thinner Aδ size (2–5 μm). Mechano-nociceptive Mrgprd-IR was *only* found in peripherin-IR axons.

Conclusions: Complex somatosensory receptors in rat molars include two types of dentinal endings that both differ from CGRP-IR endings, and at least two newly defined types of pulpal endings. The PV-IR neurons with their widely branched, synaptophysin-rich, intradentinal beaded endings are good candidates for endodontic non-nociceptive, low threshold, unperceived mechanoreceptors. The complex molar dentinal and pulpal sensory systems were not found in rat incisors.

1. Introduction

Teeth are densely innervated and their innervation responds both to noxious and mechanical stimuli (e.g., Anderson, Hannam, & Matthews, 1970; Dong, Chudler, & Martin, 1985; Fried, Sessle, & Devor, 2011; Närhi, 1985). Tooth pain is the main intradental sensation, with unperceived mechanosensitivity helping to protect teeth from damage by triggering jaw-opening reflexes. However, there is also evidence for *intra*dental, low threshold, mechanosensory, non-painful systems that could provide elaborate unperceived proprioception during normal dental functions such as chewing, biting, gritting, grinding, grasping, or bruxing (Dong & Chudler, 1984; Dong et al., 1985; Dong, Shiwaku, Kawakami, & Chudler, 1993; Olgart, Gazelius, & Sundstrom,1988) via Aδ and Aβ fast-conducting somatosensory axons (Cadden, Lisney, & Matthews, 1983; Chudler, Dong & Kawakami, 1985; Dong et al., 1985, 1993; Kubo et al., 2008). Many of those neurons respond vigorously to tooth vibration and also can provide texture discrimination (Dong et al., 1993; Paphangkorakit & Osborn, 1998; Robertson, Levy, Petrisor, Lilly, & Dong, 2003), thereby requiring low-threshold, intradental mechanisms. Tingling or vibration can be elicited by low intensity dental stimulation in people without activation of periodontal nerves or pain (Brown, Beeler, Kloka, & Fields, 1985; McGrath, Gracely, Dubner, & Heft, 1983; Osborn, 1997, 1998; Osborn, 1997, 1998; Robertson et al., 2003). Large myelinated axons occur in many kinds of mature adult teeth (Byers & Dong, 1983; Fried & Hildebrand, 1981; Gunji, 1982; Maeda, Iwanaga, Fujita, Takahashi, & Kobayashi, 1987;

Abbreviations: myelinated: Aβ, 5–10 μm diameter: Aδ, 2–5 μm, Axons; CGRP, calcitonin gene related peptide; C, coiled receptor; F, Fork shaped; GDNF, glial derived neurotrophic factor; Gfrα1, GFR, GDNF receptor; p75, low affinity neurotrophin receptor; IR, immuno-reactivity; L, layered sensory arrays; Mrgprd, MAS-related g-protein receptor type-d; NFP, neurofilament protein; PV, parvalbumin; PER, peripherin; Horizontal, PxH, Tangled, PxT, vertical, V, plexus shapes; SYN, synaptophysin; Th, thick endings; WB, widely branched beaded endings

* Corresponding author.
E-mail address: byersm@uw.edu (M.R. Byers).

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Naftel, Bernanke, & Qian, 1994), and a variety of dental receptor mechanisms have been proposed (e.g. Alavi, Dubyak, & Burnstock, 2001; Brännström & Aström, 1972; Dong et al., 1985; Farahani, Simonian, & Hunter, 2011; Fried, Sessle, & Devor, 2011; Gunji, 1982; Henry, Luo, & Levinson, 2012; Hermanstyne, Markowitz, Fan, & Gold, 2008; Hildebrand, Fried, Tuisku, & Johansson, 1995; Kvinnsland et al., 2004; Li, Ikeda, & Suda, 2013; Maeda, Iwanaga, Fujita, & Kobayashi, 1986; Magloire, Couble, Thivichon-Prince, Maurin, & Bleicher, 2009; Nweeia et al., 2014; Olgart et al., 1988; Silverman & Kruger, 1987). In particular, large fast, low threshold, mechanosensory neurons in normal cat canines have physiological properties similar to vibration-detecting Pacinian corpuscles, and they have pathways into brainstem and SI-somatosensory cortex areas that suggest non-painful functions (Dong and Chudler, 1984; Dong et al., 1985; , 1993; Dong, Chudler, & Kawakami 1990) with similar findings in monkeys (Chudler et al., 1985) and humans (Kubo et al., 2008).

We have used serial sections in sagittal and transverse planes, as well as a variety of antibodies for more complete definition of the structural and neurochemical details of complex receptor endings in mature rat molars. Seven antibodies for key molecules that have been found in subgroups of dental innervation were selected: (1) **peripherin**, an intermediate filament in small to large dental axons (Veerayutthwilai, Luis, Crumpton, MacDonald, & Byers, 2006); (2) **parvalbumin**, a calcium binding protein that has been found in human and rat teeth (Ichikawa, Ueyama, Yamaai, Sugimoto, & Matsumura, 1994; Ichikawa, Deguchi et al., 1994; Ichikawa, Deguchi, Nakago, Jacobowitz, & Sugimoto, 1995) and in mechanoreceptors (del Valle, Cobo, Cobo, & Vega, 2012); (3) **neurofilament protein**, that labels the Aβ and Aδ sensory axons (Alavi et al., 2001; Henry et al., 2012; Maeda et al., 1986, 1987); (4) **synaptophysin**, that shows sites of exocytosis via SNAP-SNARE mechanisms (Norlin, Hilligas, & Brodin, 1999); (5) **neuropeptide CGRP**, that is found in dental Aδ and C-fibers (Berggreen & Heyeraas, 2000; Byers & Närhi, 1999; Silverman & Kruger, 1987; Wakisaka et al., 1987); (6) **neurotrophin receptors, that bind the growth factors NGF or GDNF** (Byers, Wheeler, & Bothwell, 1992; Kvinnsland et al., 2004; Matsuo et al., 2001; Mitsiadis, Magloire, & Padilla, 2017; Pan, Naftel, & Wheeler, 2000; Yang, Bernanke, & Naftel, 2006), and (7) **MAS-related-g-protein receptor type-d (Mrgprd),** that reveals mechanonociceptive neurons in the peripheral nervous system (Rau et al., 2009; Zylka, Rice, & Anderson, 2005) including in rodent teeth (Chung, Jue, & Dong, 2012).

Molar crown pulp shape, root closure, and sensory innervation have matured in rats by three months compared to immature forms in young adults (Byers & Westenbroek, 2011; Naftel et al., 1994; Veerayutthwilai et al., 2006), and so we concentrated here on rats that were 2.5–4 months old, with qualitative comparisons to molars of a few older rats at 6–10 months and to the continually erupting incisors. Our goals were (a) to define receptor systems containing parvalbumin or peripherin that end in dentin and their differences from neuropeptide CGRP-IR dentinal innervation, (b) to analyze complex receptors that end in pulp and their special relationships with the plexus of Raschkow, and (c) to compare rat molars with rat incisors.

2. Materials & methods

2.1. Animals

These studies were approved by the Animal Care and Use Committee of the University of Washington and follow all ethical guidelines of the National Academies for animal research (8th Ed., 2011, www.nap.org). Adult Sprague-Dawley rats (Charles River, Hollister, CA) adjusted to the vivarium for 4–5 days. They were provided with food and water *ad libitum* plus cardboard tunnels for environmental enrichment, 12 h. light/dark cycle, and normal temperature and humidity. Prior to euthanasia the rats were mostly sleeping for 8–10 h, with access to water but not food. Detailed studies included adult males at ages 10–12 weeks (N = 19, 319–389 g) and 3.5–4 months (N = 5, 469–510 g), in which the two ages had similar molar shapes and innervation. Those were compared qualitatively with three post-maternal females at 3–4 months old (263–304 g) and older males at 6–10 months (N = 4, 580–640 g).

Table 1
Antibody Reagents.

Antibodies	Supplier (concentration)	Dilution
Primary Antibodies (code)		
CGRP MC (C7113)	Sigma-Aldrich (2.0 mg/ml)	1:150
GDNF receptor GFRα1 PC (H-70)	Santa Cruz (0.2 mg/ml)	1:60
MrgprD PC (AMR-061)	Alomone (0.8 mg/ml)	1:700
Neurofilament 200 MC (N52)	Sigma-Aldrich (11 mg/ml)	1:200
Parvalbumin PC (PA1-933)	Life Tech (0.1 mg/0.1 ml)	1:250
Parvalbumin MC (PARV-19)	Sigma-Aldrich (1.25 mg/ml)	1:200
Peripherin PC (AB1530)	Chemicon (0.1 mg/ml)	1:800
Peripherin MC (8G2)	Sigma-Aldrich (1.0 mg/ml)	1:100
p75 Neurotr. receptor MC (192)	M. Bothwell[a] (ascites)	1:500
Synaptophysin PC (Z66)	Zymed (0.1 mg/ml)	1:750
Secondary Antibodies		
Goat anti-Rabbit, biotinylated[b]	Vector (BA-1000)	1:500
Horse anti-mouse, biotinylated[b]	Vector (BA-2001)	1:500
Donkey anti-rabbit (Alexa 488)[c]	Molecular Probes/Life Tech	1:600
Goat anti-mouse (Alexa 555)[d]	Molecular Probes/Life Tech	1:500

[a] We thank Dr. Mark Bothwell, University of Washington, for this monoclonal antibody to low affinity p75 neurotrophin receptor.
[b] for ABC perioxidase reaction with nickel-enhanced DAB (Black).
[c] GREEN fluorescence to detect polyclonal (PC) antibodies.
[d] RED fluorescence to detect monoclonal (MC) antibodies.

2.2. Anesthesia, fixation, decalcification, sectioning

Initial sedation with ketamine (55 mg/kg), Xylazine (5.5 mg/kg), and acepromazine (1.1 mg/kg) in saline occurred at late afternoon and was followed by an overdose of 'Beuthanasia-D' (Schering-Plough NADA#119-807, 85 mg/kg pentobarbital) in 0.1 M phosphate buffer (PB). As soon as paw reflexes were absent and respiration was shallow, rats were perfused through the heart with 4% formaldehyde in 0.1 M PB (pH7.4) for 8 min, followed by cooling for 30 min, dissection of jaw tissues and trigeminal ganglia, and post-fixation for 3–4 h. Jaws were decalcified with ethylenediaminetetraacetic acid (10% EDTA in water, pH7.4) that was changed 2–3 ×/week for 1 month, followed by weekly EDTA changes for another 1–3 months. Cryoprotected jaws (30% sucrose in PB) were serial sectioned at 40 μm (transverse or sagittal planes), and immunoreacted for key proteins (Table 1) at a section interval of 1:4 per antibody (i.e. with 120 μm between sections) and with a random start for each serial set. For DAB (Black) histochemistry (Figs. 1–4), we used monoclonal (MC) antibodies for neurofilament protein, parvalbumin or peripherin, and polyclonal (PC) antibodies for parvalbumin, peripherin and synaptophysin. For fluorescence double labeling, we used MC (RED) and PC (GREEN) primary antibody combinations, as indicated in each figure (Figs. 5–11) and Table 1.

2.3. Diaminobenzedine immunohistochemistry

Sections were rinsed in 0.1 M phosphate buffered saline (PBS), blocked in vehicle (PBS plus 0.2% Triton-X-100 plus sera at 2.0–2.5% each), and incubated overnight in primary antibody (Table 1). Second antibodies (2 μl/ml vehicle, biotinylated goat anti-rabbit or horse anti-mouse, Vector) were incubated 3–4 h, rinsed, incubated 2 h in avidin-biotin complex (ABC peroxidase, Vector) followed by tris buffered saline (TBS) rinses and incubation in 0.05% DAB (Sigma) in TBS with 0.2% nickel ammonium sulfate enhancement. Sections were rinsed, mounted on gelatin-coated slides, dried, covered with permount, and examined with Nikon bright-field optics. All images were observed at 300 dpi and had similar illumination and leveling (Adobe Photoshop