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# Dental pulp and gingivomucosa in rats are innervated by two morphologically and neurochemically different populations of nociceptors

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ABSTRACT

*Objectives:* Difference in phenotypes of sensory neurons innervating dental pulp or gingivomucosa may be responsible for intense pain sensations in pulpitis in contrast to relatively painless chronic periodontitis. Therefore, we classified these neurons according to their size and two neurochemical characteristics of nociceptors, their TrkA expression and isolectin IB4 binding.

*Design:* In rats ($n = 6$) fluorescent tracers Fluorogold and TrueBlue were simultaneously applied into the standard-sized tooth cavity and nearby gingival sulcus, respectively. After the fluorescence on paraffin trigeminal ganglia (TG) sections was identified and photographed, immunohistochemistry for TrkA expression and IB4 binding was performed on the same sections.

*Results:* The average sizes of TG neurons projecting to the gingivomucosa and dental pulp were $894 \pm 441$ $\mu m^2$ and $1012 \pm 381$ $\mu m^2$, respectively. The proportions of small-sized gingival and pulpal neurons were 14% and 5%, respectively ($p < 0.05$). The proportions of TrkA-positive neurons among all gingival or pulpal neurons were 76% and 86%, respectively ($p < 0.05$). Among all gingival or pulpal neurons the proportions of IB4-positive neurons were 46% and 3% ($p < 0.001$), respectively, and the majority of them were small-medium sized.

*Conclusions:* Dental pulp and gingivomucosa are richly innervated by nociceptive TrkA-expressing neurons. However, while great majority of pulpal neurons are larger NGF-dependent A-fibre nociceptors without affinity to bind IB4, almost half of the gingival neurons are smaller IB4 binding C-fibre nociceptors. The difference in phenotype of sensory neurons might partially explain the different sensitivity of both tissues during normal and pathological conditions.



## 1. Introduction

The generation of dental pain results from activation of primary afferent neurons in the trigeminal ganglion (TG) with peripheral axons that project to dental pulp or periodontal tissue. Primary sensory neurons can be classified into various subpopulations based on their morphology as well as their physiologic and neurochemical characteristics. In general, they encompass large-diameter sensory neurons with

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predominately thick myelinated axons which in physiologic conditions do not convey noxious input, medium-diameter neurons with predominately thin myelinated (Aδ) axons and small-diameter sensory neurons with predominately unmyelinated (C) axons. According to their neurochemical characteristics, the medium and small-diameter nociceptive neurons can be further subdivided into three main populations: small-diameter peptidergic neurons that express neuropeptides, such as calcitonin gene-related peptide (CGRP) and substance P (SP), medium-sized peptide (CGRP) expressing neurons and small-diameter nonpeptidergic neurons.[1,2] Another classification divides nociceptive neurons into two partially overlapping populations.[3,4] The neurons of one population, which are predominantly peptidergic, express TrkA receptor for neurotrophin nerve growth factor (NGF)[1,5,6] while neurons from other population, predominantly nonpeptidergic, bind isolectin B4 (IB4) from plant *Griffonia simplicifolia*, and have predominantly unmyelinated C-fibres.[5–9] The same authors suggested the existence of the third subgroup encompassing about one-third of the neurons with C-fibres that simultaneously express TrkA and bind IB4, probably also expressing and secreting neuropeptides.[3,4] This classification seems important as the nature of the pain depends on functional differences between these three types of nociceptors, both after acute or prolonged noxious stimulus. IB4-binding neurons have longer action potentials, slower conduction velocities and more negative membrane potentials than neurons which do not bind IB4, which is all related to expression of Nav1.9 channel on their cell membrane.[10] They are projected to more superficial targets (e.g. skin) in contrast to the neurons with deeper afferents (e.g. joint) that do not bind IB4.[4] IB4 binding dorsal root ganglion (DRG) neurons also project to different central pathways (lamina II inner) than TrkA expressing DRG neurons (projection to laminas I and II outer), indicating also anatomical and functional difference in their central pathways.[10] However, it is not known yet into which pathways the neurons that simultaneously bind IB4 and express TrkA are projected.

It has been shown that all DRG neurons which express TrkA are nociceptive, regardless of their cell sizes.[3] About 40–50% of the DRG or TG neurons in adult rat express the TrkA receptor, the majority of them having small or medium sized perikarions.[11–14] Among TG neurons innervating the rat gingivomucosa, approximately 70% contain the TrkA receptor,[14] which corresponds well with the proportions of the neurons that express neuropeptides CGRP and SP.[15] Most pulpal afferent neurons have cytochemical features of nociceptors that express neuropeptides and respond to NGF.[16]

In DRGs, nociceptive neurons with the ability to bind IB4 normally account for about one third of the DRG neurons predominately belonging to the cutaneous afferent neurons.[17] Only few TG neurons projecting to the dental pulp bound the lectin IB4.[18] The proportions and the morphological properties of the IB4-binding neurons in TG that project to the gingiva have not been evaluated yet.

It has been established that chronic periodontitis is usually a non-painful disease[19] in contrast to pulpitis, which is characterized by intense pain sensations.[20] The differences between the mechanisms of intense pain sensations of normal pulp or during pulpitis and the mechanisms of relatively painless, but destructive, chronic periodontitis are still not completely characterized. The reason for this may to same extent lie in the existence of different phenotypes of sensory neurons that innervate these two oral tissues. It is interesting that studies directly comparing the morphometry and the nociceptive phenotype of TG neurons projecting to dental pulp and periodontal tissue are rare. Therefore, in this study we decided to simultaneously identify the sensory neurons which innervate the dental pulp and corresponding nearby gingivomucosa in rats, by using two different retrograde neuron labelling substances applied to the pulpal or gingivomucosal tissue, respectively. Thereafter, we classified these neurons according to their morphometric properties and in regard to two novel neurochemical characteristics of nociceptors, their TrkA expression and IB4 binding.

## 2. Materials and methods

Eight ($n = 8$) female Wistar rats (250 g) were used in experiments. All efforts were made to minimize the number of the experimental animals and their suffering. The Veterinary Administration of the Republic of Slovenia approved the experiments (The No. of the approval: 007-20-22/09). All procedures were performed under deep anaesthesia with a mixture of dihydrothyazine (Rompun, Bayer, AG, Leverkusen, Germany, 8 mg/kg; i.p.) and ketamine hydrochloride (Ketalar, Parke-Davis GmbH, Berlin, Germany, 60 m/kg; i.p.).

The rats were anaesthetized and positioned with mouth held open by micro-dissecting retractor under a surgical microscope. In 6 animals ($n = 6$), standard-size cavities were made on the occlusal surfaces of the right first maxillary molars with a high speed hand-piece and a ¼ round bur until the pulp chamber was exposed. Verification of the pulp exposure was made with a probe (tactile ''give'' of the tooth) and by visualization of bleeding from the bottom of the cavity. 1 μl of 2% aqueous solution of Fluorogold (FG) was applied into the cavity and left to absorb for 10 min. Cavities were closed with Cavit® (Densply, Germany). Afterwards, TrueBlue (TB) crystals were carefully applied into the palatinal and buccal gingival sulcus of the same tooth. In a separate experiment ($n = 2$) we evaluated the distribution of TB in the gingivomucosa 2 and 5 days after TB application. Gingivomucosal tissue around the first molar tooth was dissected from these two animals and prepared for conventional histology. After 10 μm thick paraffin sections were photographed under a fluorescence microscope, the same sections were counterstained with haematoxylin and eosin (see Fig. 1).

Other six animals were sacrificed 7 days after FG and TB application. TGs were dissected, immersion fixed in 10% formalin and embedded in paraffin. Every third and fourth histological sections, 6 μm thick, were collected for a further analysis of TrkA and IB4 binding, respectively. Before deparaffinization, TB and FG fluorescences were photographed under a fluorescence microscope (Olympus, IX81, Japan) and later these photos were used to be compared with images obtained with a light microscope. Subsequently, third and fourth series of histological sections were used for TrkA immunohistochemistry using anti-rat TrkA antibody