VR1- and VRL-1-like immunoreactivity in normal and injured trigeminal dental primary sensory neurons of the rat

Emma Stenholm, Ulf Bongenhielm, Michael Ahlquist and Kaj Fried

Department of Neuroscience, Karolinska Institutet, Stockholm, Sweden; Department of Odontology, Huddinge Hospital, Karolinska Institutet, Huddinge, Sweden

Stenholm E, Bongenhielm U, Ahlquist M, Fried K. VR1- and VRL-1-like immunoreactivity in normal and injured trigeminal dental primary sensory neurons of the rat. Acta Odontol Scand 2002:60:72–79. Oslo. ISSN 0001-6357.

The vanilloid receptor VR1 and the vanilloid receptor-like protein VRL-1 are associated with polymodal nociceptors, and may be important for pain processing in normal and injured teeth. Using immunohistochemistry, we have studied the distribution of these receptors in rat pulpal or gingival trigeminal ganglion neurons that were identified through retrograde labeling with fluoro-gold. Twenty-one percent to 34% of tooth pulp-innervating neurons were VR1-positive, while 32%–51% were VRL-1-immunoreactive. However, double-labeling experiments revealed that VR1 and VRL-1 rarely co-existed in the same cells, but rather seemed to be confined to separate subpopulations. Among the gingival neurons, about 25% were VR1-positive and about 41% were VRL-1-immunoreactive. A lesion of the inferior alveolar nerve, which supplies mandibular teeth and gingiva, resulted in a marked down-regulation of VR1 in the affected trigeminal ganglion cells. A down-regulation of VRL-1 was also indicated. The results suggest that both VR1 and VRL-1 could have significant roles in pulpal and gingival nociceptive transduction. □ *Gingiva; immunohistochemistry; tooth pulp; trigeminal ganglion; VR1; VRL-1, nerve injury*

E. Stenholm, Centre for Oral Biology, Novum, Karolinska Institutet, P.O. Box 4064, SE-141 04 Huddinge, Sweden. Fax: +46 8 779 3166, e-mail: Emma.Stenholm@ofa.ki.se

Mammalian tooth pulps have what is believed to be a purely nociceptive sensory innervation derived from primary sensory neurons located in the trigeminal ganglion. Some sympathetic axons reach the teeth by following the external carotid artery and its branches (see ref. 1). Dentinal pain can be elicited by a variety of stimuli, but the tooth pulp appears to be insensitive to exteroceptive stimulation, and it is only in pathological conditions that it becomes sensitive to relatively mild direct stimulation. As revealed in numerous studies, electrical thermal, mechanical and chemical stimulation of the tooth pulp all result in the subjective experience of pain. Many small, pain-transducing sensory neurons in general are activated by capsaicin, a vanilloid. This causes a transient pain, but if the exposure to capsaicin is prolonged, the nerve is inactivated and analgesia follows (see refs. 2, 3). The capsaicin receptor VR1 causes an increase in cation conductance which is selective for calcium ions (4, 5). The VR1 receptor is also a physiological transducer for painful heat. It is activated at temperatures above 43°C (see 3, 6). Another receptor that is structurally related to VR1, the VRL-1 receptor, has recently been identified (7). However, although it has a structure similar to VR1, it is insensitive to capsaicin. Instead, it is sensitive to heat, although it is activated at a higher threshold than VR1, at 52°C (6, 7). The possible involvement of vanilloid receptors in pulpal pain mechanisms has not received much attention. The purpose of this study was therefore to examine the expression of VR1 and VRL-1 in trigeminal neurons that were identified as tooth pulp- or gingiva-innervating by means of retrograde tracing with fluoro-gold. Furthermore, we examined the effect of nerve injury on VR1- and VRL-1-expression in trigeminal neurons related to dental targets. While this study was in progress, immunohistochemical reports have appeared on VR1 and VRL-1 expression in intact trigeminal tooth pulp neurons (8, 9). In addition, a functional study using patch clamp analysis and RT-PCR has just recently demonstrated that dissociated tooth pulp-innervating neurons respond to capsaicin and express VR1 mRNA (10).

Material and methods

Ten adult male Sprague-Dawley rats were used in this study. In 4 animals under general anesthesia (Chloral hydrate 350 mg/kg, i.p.) the inferior alveolar nerve (IAN) was exposed via an incision along the lateral surface of the mandibular ramus. Bone overlying the mandibular canal was removed and the nerve exposed a few millimeters below the mandibular incisure. The nerve was carefully separated from the surrounding vasculature. The IAN was then tightly ligated with a 6/0 silk suture and cut immediately distal to the ligature. Immediately proximal to the ligature, an injection of 0.1 μl 1% fluorogold (FG; Fluorochrom, Englewood, Colo., USA) was performed. The incision was then closed. In 2 animals an injection of 0.1 μl FG was performed in the gingiva associated with the first molar in the lower jaw. In 4 animals, a cavity was drilled in the occlusal part of the 1st maxillary molar. The cavity was injected with 0.1–0.2 μl FG, and sealed with IRM. Three days after FG injections, all 8 animals were deeply anesthetized as above, and perfused through the ascending aorta with Tyrode's solution (37°C) followed by

Taylor&Francis healthsciences

© 2002 Taylor & Francis

4% formaldehyde in a 0.1M phosphate buffer containing 0.2% picric acid (4°C). The trigeminal ganglion on the side of the injection was removed. For controls of immunostaining, the corresponding intact ganglia were removed. All specimens were post-fixed in the fixative for 90 min, immersed in 0.1M buffer with 10% sucrose overnight (4°C) and rapidly frozen. Serial longitudinal sections (14 μm) throughout all ganglia were cut on a cryostat (Microm, Germany) and mounted on gelatin/chrom alum coated object slides. The sections were incubated for 24 h in a humid atmosphere at 4°C with guinea pig polyclonal antibodies against the VR1 receptor (dilution 1:1000; Neuromics, Minneapolis, Minn., USA) or with rabbit polyclonal antibodies against the VRL-1 receptor (dilution 1:400; Chemicon, Temecula, Calif., USA). For double-labeling, some sections were incubated with a mixture of the VR1 and the VRL-1 antisera, or with a mixture of the VR1 antiserum and rabbit polyclonal antibodies against calcitonin gene-related peptide (CGRP) (dilution 1:400; Peninsula, Belmont, Calif., USA). After rinsing with 0.01M PBS the sections were incubated with Cy3-conjugated donkey anti-guinea pig antiserum (diluted 1:250, Jackson Immuno Research, USA) or with Cy3-conjugated donkey anti-rabbit antiserum (diluted 1:250, Jackson Immuno Research, USA) in a humid atmosphere at room temperature for 1 h. Double-labeled sections were incubated with a mixture of the Cy3-conjugated donkey anti-guinea pig antiserum and Cy2-conjugated donkey anti-rabbit antiserum (diluted 1:250, Jackson Immuno Research, USA). All antisera were diluted in 0.01M PBS containing 0.3% Triton X-100, 5% bovine serum albumin (BSA), 3% normal donkey serum, and 0.1% sodium azide. Control sections were incubated either using the preabsorption test in which the primary antibody was mixed with the corresponding peptide (VR1: 10-fold excess by weight, Neuromics, Minneapolis, Minn , USA; not performed with VRL-1 antiserum since a peptide was unavailable), or by omitting the primary antibody. Both these procedures abolished immunostaining. Subsequently, the sections were rinsed in 0.01M PBS, mounted in a mixture of glycerol and 0.01M PBS (3:1), coverslipped, and examined in a Leitz DM RBE fluorescence microscope equipped with proper filter combinations. Measurements of neuronal soma area were performed using an image analyzing system equipped with a computer-based video camera (software: Image 1.28, National Technical Information Service, Bethesda, Md., USA). Photographs were taken with a Nikon CoolPix 950 digital camera, digitally processed and printed.

Results

Tooth pulp

Three days after FG injections into upper jaw molars, FG-labeled neurons were located exclusively in the maxillary division of the trigeminal ganglion. These cells had areas ranging in size between 100 and ~3000 μm^2, with a peak at about 600 μm^2. VR1-like immunoreactive (IR) FG-positive cells had sizes between 100 and 1600 μm^2, while VRL-1-IR pulpal neurons were between 150 and about 3000 μm^2 (Fig. 1A, B). The proportion of VR1-positive FG cells varied between 21% and 34 %, while between 32% and 51% of all FG neurons showed VRL-1-IR (Figs. 2, 3A, B and 4A, B). However, double-labeling experiments revealed that VR1 and VRL-1 rarely co-existed in the same cells, but rather seemed to be confined to separate subpopulations. Furthermore, double-labeling also showed that a majority of the VR1 cells (~88%) coexisted with CGRP (Fig. 5).

Gingiva

After FG injections into the mandibular gingiva, FG-labeled neurons were located in the mandibular division of the trigeminal ganglion. The cells had areas ranging in size between 100 and 2000 μm^2, with a peak at 700 μm^2. VR1-IR FG-positive cells had sizes between 100 and 1500 μm^2, while VRL-1-IR gingival neurons were between 100 and 2000 μm^2 (Fig. 1C, D). The proportion of VR1-positive FG cells varied between 21% and 26%, while between 41% and 42% of all FG neurons showed VRL-1-IR (Figs. 2C, D, 3C, D and 4C, D).

IAN injury

Three days after IAN axotomy and FG injection into the proximal nerve stump, a large number of FG-labeled neurons were located in the mandibular division of the trigeminal ganglion. These cells had areas ranging in size between 100 and ~2000 μm^2, with a peak at about 600 μm^2. Only very few VR1-IR FG-positive cells were encountered, and these had sizes between 200 and 1000 μm^2, while VRL-1-IR neurons were between 300 and about 1400 μm^2 (Fig. 1E, F). After IAN injury, 0.3%–1.5% of all FG-positive trigeminal mandibular neurons were VR1-positive, while about 10% (7.4%–12.7%) of all FG neurons showed VRL-1-IR (Figs. 2E, F, 3E, F and 4E, F).

Discussion

Here we show that both VR1 and VRL-1 are expressed in tooth pulp-innervating ggl V neurons as well as in gingival trigeminal ganglion neurons. As previously reported in studies of the dorsal root and trigeminal ganglia of rats, VR1-IR was restricted to small and medium-sized neurons (11). Furthermore, we demonstrate that axotomy of the IAN causes a down-regulation of VR1 and VRL-1 IR in mandibular neurons of the trigeminal ganglion (see below). While this study was under completion, Ichikawa & Sugimoto (8, 9) reported on VR1 and VRL-1 expression in pulpal neurons, using an approach similar to the one employed here. In accordance with our results, both

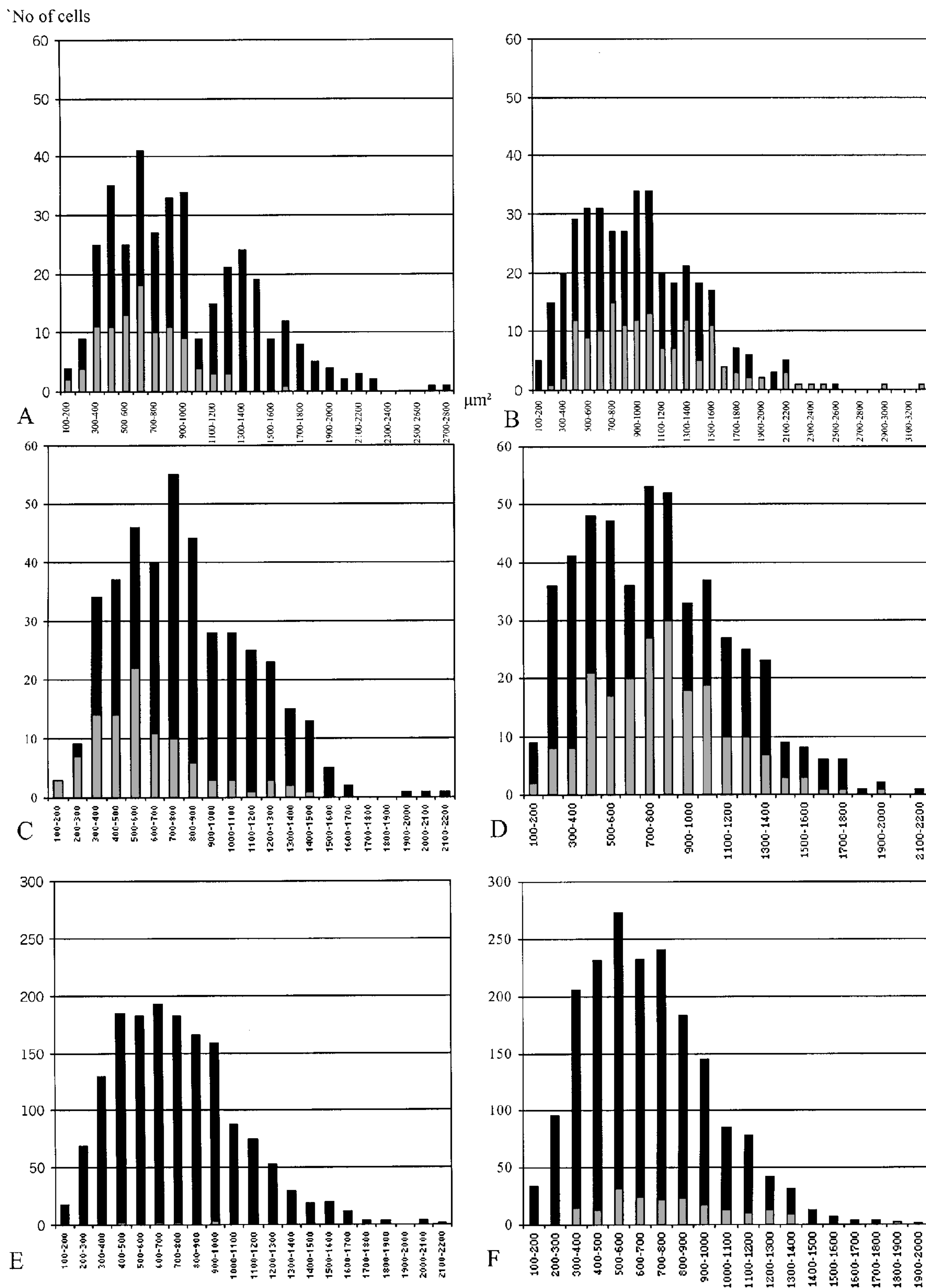

Fig. 1. Histograms showing the size distribution (area) of FG-labeled VR1- (A, C, E) or VRL-1 (B, D, F)-positive trigeminal ganglion neurons belonging to the tooth pulp (A, B), gingiva (C, D), or injured IAN (E, F). Each histogram represents pooled data from 4 rats (A, B and E, F) or 2 rats (C, D). Black bars show sizes of all FG-labeled neurons that were sampled and grey bars depict the proportion of immunoreactive neurons.

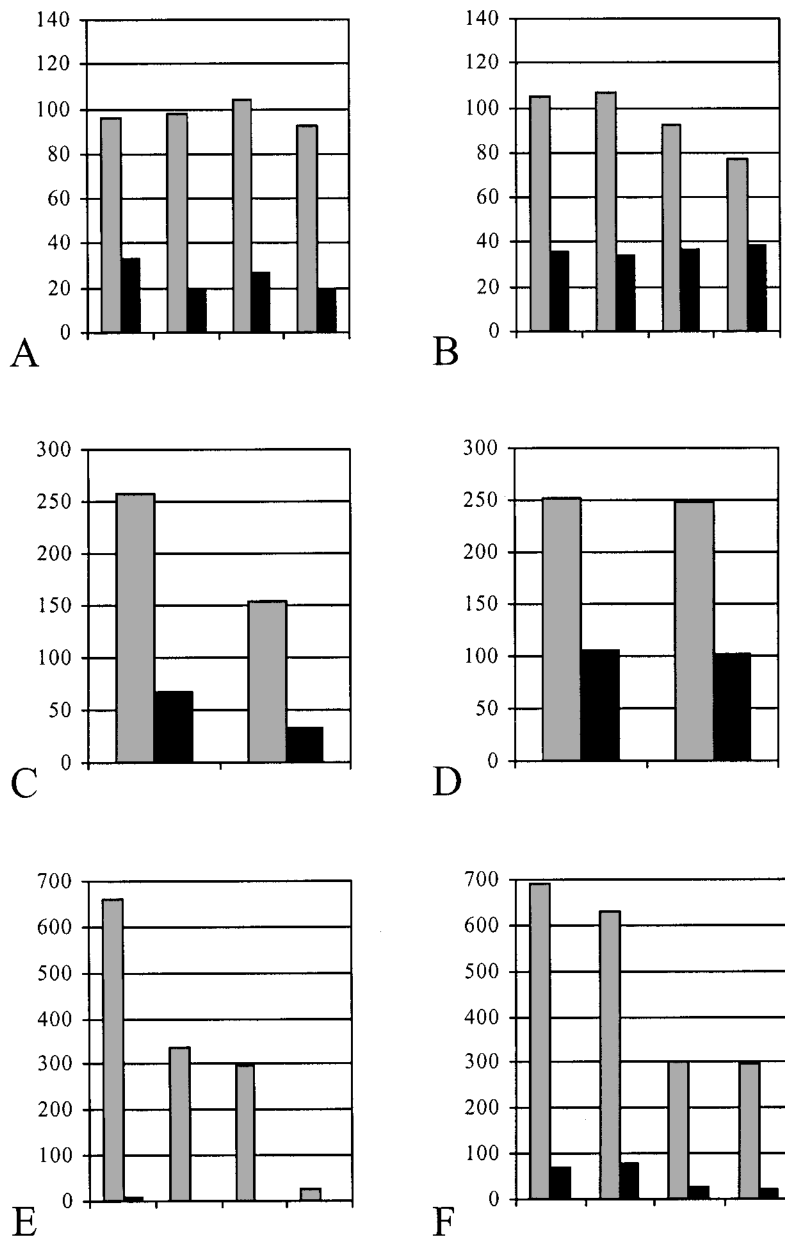

Fig. 2. Diagrams showing the proportion of VR1- (A, C, E) or VRL-1-(B, D, F) positive FG-labeled neurons in the trigeminal ganglion after retrograde labeling from the tooth pulp (A, B, 4 rats), gingiva (C, D, 2 rats) or injured IAN (E, F, 4 rats). Each case is represented by a pair of bars, where the grey bar represents total number (y-axis) of FG-positive neurons counted in one ganglion, and the black bar the number of these neurons that were VR1- or VRL-positive.

vanilloid receptors were found to be present in tooth-pulp innervating neurons. This confirms that a group of pulpal neurons are capsaicin-sensitive and respond to noxious heat. However, although the findings on VRL-1 IR are similar, the proportion of VR1 neurons seem to differ from those we obtained. Thus, while we found 32%–51% VRL-1-IR and 21%–34% VR1-positive pulpal neurons, the corresponding figures in the studies by Ichikawa & Sugimoto (8, 9) were 37% and 8%, respectively. We have at present no apparent explanation for the discrepancy in VR1 labeling. However, in the elegant patch-clamp study by Chaudhary et al. (10), it was found that 65% of tooth pulp-innervating trigeminal neurons responded to capsaicin. This indicates that a significant, rather than a marginal, proportion of dental primary sensory neurons express the VR1 receptor. Studies of the properties of the capsaicin-sensitive VR1 receptor have shown that it is directly gated by heat, and that protons can greatly modulate its sensitivity. Under acidic conditions, VR1 can be activated at room temperature (3, 5). Local tissue

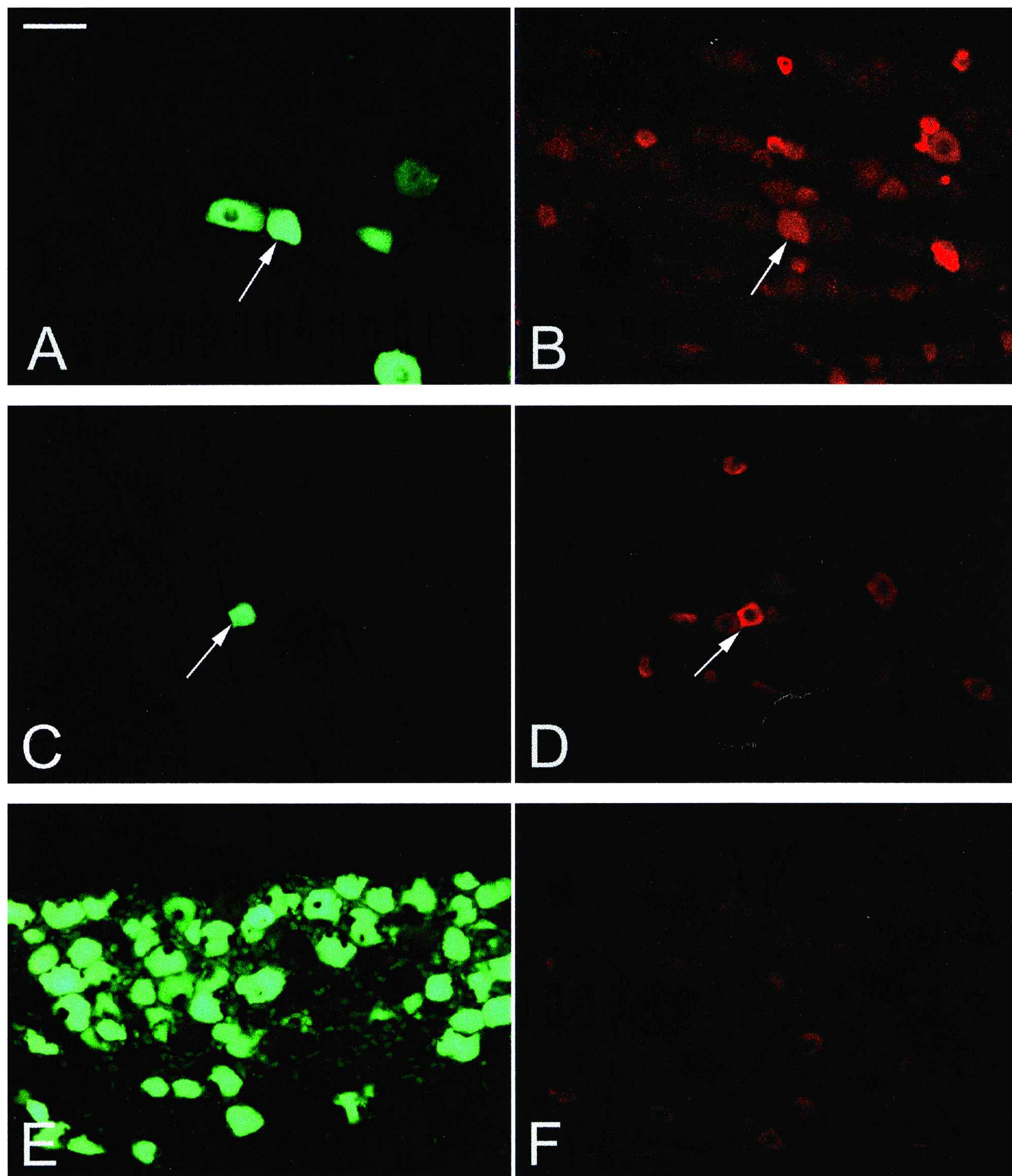

Fig. 3A–F. Fluorescence micrographs from sections of rat trigeminal ganglia retrogradely labeled with fluoro-gold from tooth pulp (A, B), gingiva (C, D), or injured IAN (E, F) and incubated with antibodies against VR1. Among the FG-labeled neurons in A and C, arrows depict pulpal (A, B) and gingival (C, D) VR1-positive nerve cells. E shows a large number of FG-labeled neurons after IAN injury, and F reveals that very few or none of these were VR1-immunoreactive. Scale bar in A (applies to A–F): 50 μm.

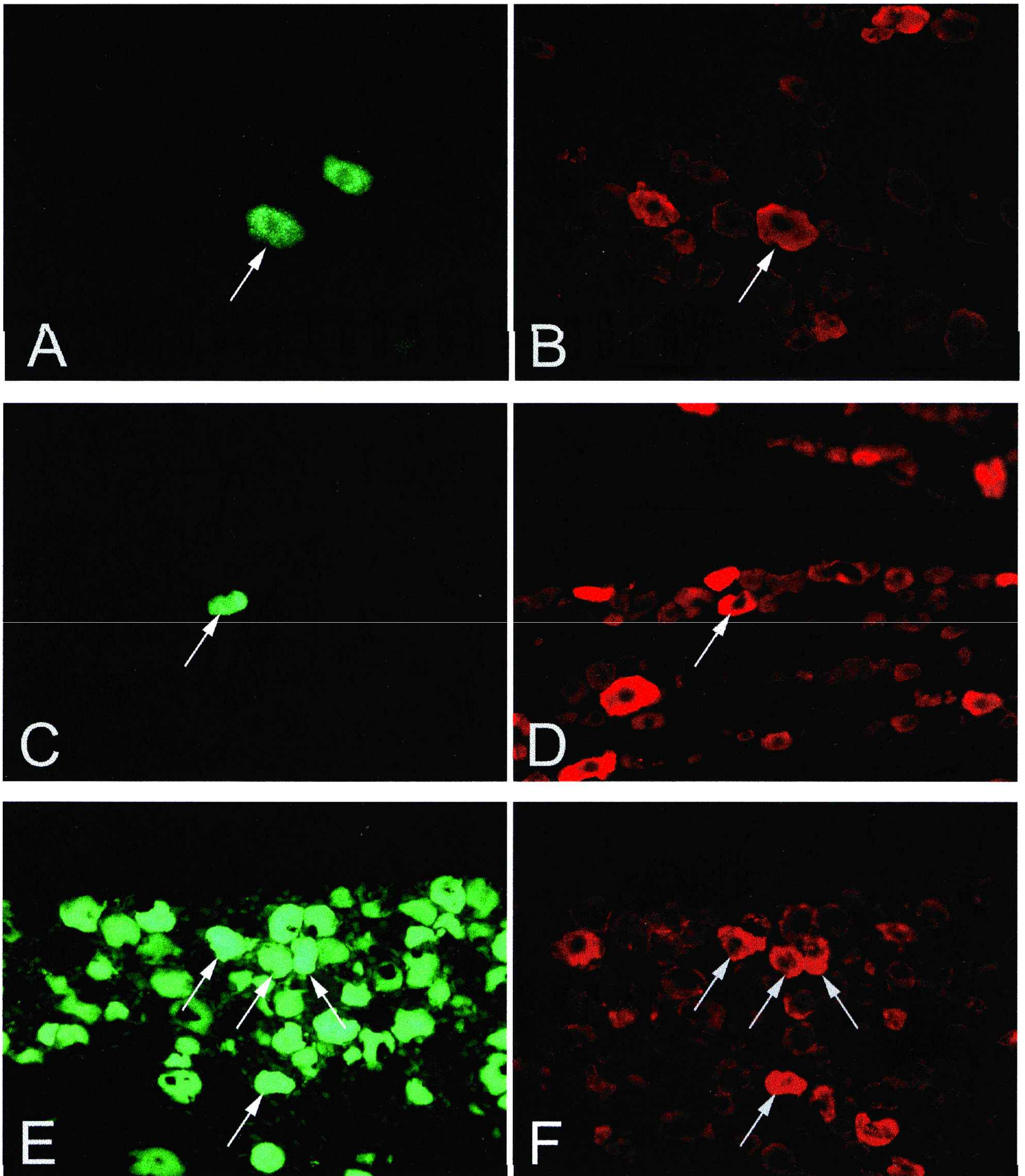

Fig. 4A–F. Fluorescence micrographs from sections of rat trigeminal ganglia retrogradely labeled with fluoro-gold from tooth pulp (A, B), gingiva (C, D), or injured IAN (E, F) and incubated with antibodies against VRL-1. Arrows show examples of FG-labeled pulpal (A), gingival (C), and injured IAN (E) neurons which are VRL-1-immunoreactive (B, D, and F, respectively). Scale bar in A (applies to A–F): 50 μm.

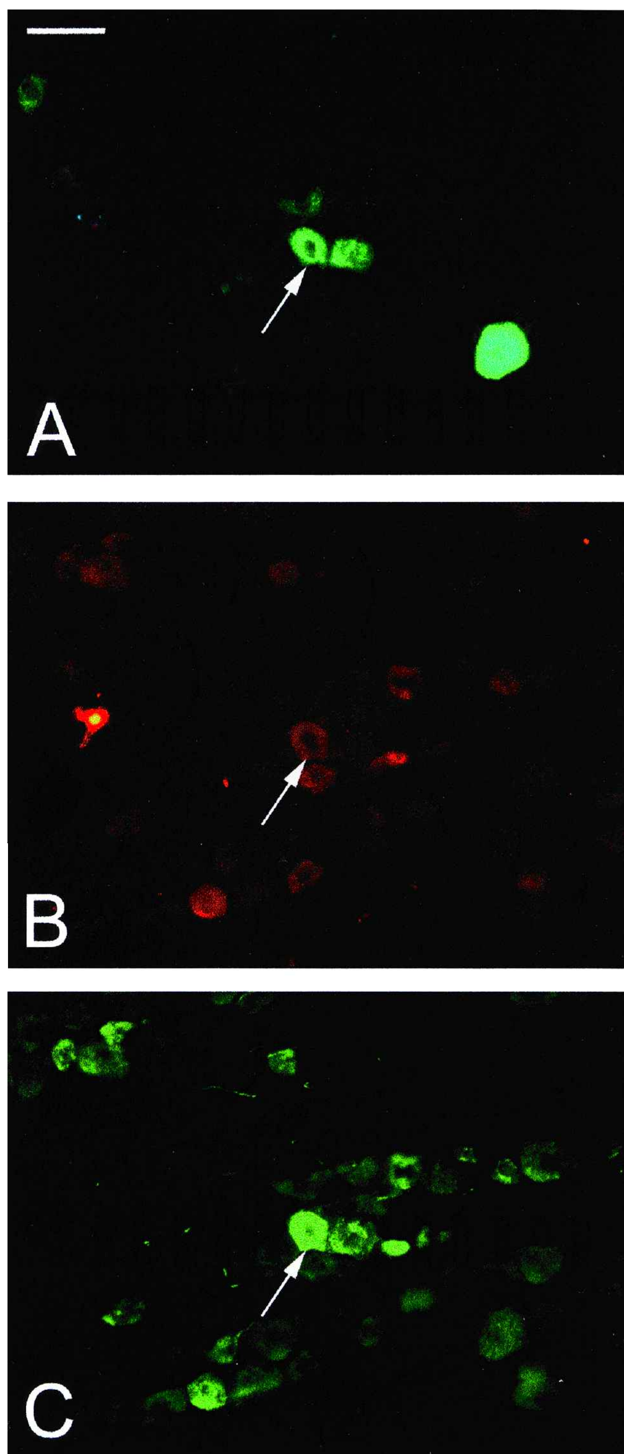

Fig. 5A–C. Fluorescence micrographs from a section of a rat trigeminal ganglia retrogradely labeled with FG from a tooth pulp (A) and incubated with antibodies against VR1 (B) and CGRP (C). The arrow shows an example of an FG-labeled pulpal neuron which is both VR1- and CGRP-immunoreactive. Scale bar in A (applies to A–C): 50 μm.

acidosis should occur frequently during pulpal inflammation, and in view of the present results it seems likely that the pain associated with this condition is to some extent conveyed by pulpal vanilloid receptors. Hence, specific blockers of these receptors could provide valuable pharmacological agents in the treatment of pulpal pain.

This paper shows for the first time that VR1 is down-regulated in affected trigeminal ganglion neurons after inferior alveolar nerve injury. The normal trigeminal ganglion contains about 50% VR1-positive neurons (11), but after IAN injury only about 1% of the damaged neurons, which dominate the mandibular part of the trigeminal ganglion (12) expressed VR1. A down-regulation of VR1 was reported in spinal DRG neurons after sciatic nerve injury (13), but considering the differences in response to injury that exist between spinal and trigeminal neurons (14), it appeared important to provide specific data on this subject from the trigeminal system. This down-regulation is probably a result of the cellular response to the injury (15), and could be secondary to a loss of trophic factors. Most likely, these factors are local or target-derived nerve growth factor (NGF) and glial cell-line derived neurotrophic factor (GDNF) (13, 16). It is not known how the nerve injury-induced plasticity in VR1 expression changes the sensitivity of trigeminal dental neurons to chemical or noxious heat stimuli. However, further knowledge on how VR1 is expressed in these neurons under pathological conditions may increase the possibility to treat associated trigeminal pain states. With regard to VRL-1, previous data on the reaction to nerve injury have not been available. However, our data indicate that this receptor is also down-regulated after axotomy. We found about 10% VRL-1-positive cells among injured IAN neurons. In the normal ganglion, at least 14% of the all neurons are VRL-1-immunoreactive (8, 9). However, intact IAN neurons probably express VRL-1 to a higher degree, since the IAN to a large extent supplies teeth and gingiva which have at least 30%–40% VRL-1-IR cells (8, present results). A down-regulation of VRL-1 may contribute to the changed properties of trigeminal nociceptors that appear after injury.

Acknowledgements.—The present report was supported by grants from the Swedish Medical Research Council (project no. 8654). We thank Ms Anita Bergstrand for her technical assistance.

References

1. Hildebrand C, Fried K, Tuisku F, Johansson CS. Teeth and tooth nerves. Progr Neurobiol 1995;145:122–65.
2. Szolcsanyi J. Actions of capsaicin on sensory neurones. In: Wood JN, editor. Capsaicin in the study of pain. London: Academic Press; 1993. p. 1–26.
3. Kress M, Zeilhofer HU. Capsaicin, protons and heat: new excitement about nociceptors. Trends Pharmacol Sci 1999;20: 112–8.
4. Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D. The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature 1997;89:816–24.

5. Tominaga M, Caterina MJ, Malmberg AB, Rosen TA, Gilbert H, Skinner K, et al. The cloned capsaicin receptor integrates multiple pain-producing stimuli. Neuron 1998;21:531–43.
6. Caterina MJ, Julius D. The vanilloid receptor: a molecular gateway to the pain pathway. Annu Rev Neurosci 2001;24:487–517.
7. Caterina MJ, Rosen TA, Tominaga M, Brake AJ, Julius D. A capsaicin-receptor homologue with a high threshold for noxious heat. Nature 1999;398:436–41.
8. Ichikawa H, Sugimoto T. VR1-immunoreactive primary sensory neurons in the rat trigeminal ganglion. Brain Res 2001;890:184–8.
9. Ichikawa H, Sugimoto T. Vanilloid receptor 1-like receptor-immunoreactive primary sensory neurons in the rat trigeminal nervous system. Neuroscience 2000;101:719–25.
10. Chaudhary P, Martenson ME, Baumann TK. Vanilloid receptor expression and capsaicin excitation of rat dental primary afferent neurons. J Dent Res 2001;80:1518–23.
11. Guo A, Vulchanova L, Wang J, Li X, Elde R. Immunocytochemical localization of the vanilloid receptor 1 (VR1): relationship to neuropeptides, the P2X3 purinoceptor and IB4 binding sites. Eur J Neurosci 1999;11:946–58.
12. Bongenhielm U, Boissonade F, Westermark A, Robinson PP, Fried K. Sympathetic nerve sprouting fails to occur in the trigeminal ganglion after peripheral nerve injury in the rat. Pain 1999;82:283–8.
13. Michael GJ, Priestley JV. Differential expression of the mRNA for the vanilloid receptor subtype 1 in cells of the adult rat dorsal root and nodose ganglia and its downregulation by axotomy. J Neurosci 1999;19:1844–54.
14. Fried K, Bongenhielm U, Boissonade F, Robinson PP. The trigeminal system and facial pain. Neuroscientist 2001;7:155–65.
15. Aldskogius H, Arvidsson J, Grant G. Axotomy-induced changes in primary sensory neurons. In: Scott SA, editor. Sensory neurons. Diversity, development and plasticity. New York: Oxford University Press; 1992, p. 363–83.
16. Ogun-Muyiwa P, Helliwell R, McIntyre P, Winter J. Glial cell line derived neurotrophic factor (GDNF) regulates VR1 and substance P in cultured sensory neurons. Neuroreport 1999;10:2107–11.

Received for publication 28 August 2001
Accepted 3 December 2001