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TOOTH PULP INFLAMMATION INCREASES BDNF EXPRESSION IN RODENT TRIGEMINAL GANGLION NEURONS

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Abstract

Nociceptive pathways with first-order neurons located in the trigeminal ganglion (TG) provide sensory innervation to the head, and are responsible for a number of common chronic pain conditions, including migraines, temporomandibular disorders and trigeminal neuralgias. Many of those conditions are associated with inflammation. Yet, the mechanisms of chronic inflammatory pain remain poorly understood. Our previous studies show that the neurotrophin brain-derived neurotrophic factor (BDNF) is expressed by adult rat TG neurons, and released from cultured newborn rat TG neurons by electrical stimulation and calcitonin gene-related peptide (CGRP), a well-established mediator of trigeminal inflammatory pain. These data suggest that BDNF plays a role in activity-dependent plasticity at first-order trigeminal synapses, including functional changes that take place in trigeminal nociceptive pathways during chronic inflammation. The present study was designed to determine the effects of peripheral inflammation, using tooth pulp inflammation as a model, on regulation of BDNF expression in TG neurons of juvenile rats and mice. Cavities were prepared in right-side maxillary first and second molars of 4-week-old animals, and left open to oral microflora. BDNF expression in right TG was compared with contralateral TG of the same animal, and with right TG of sham-operated controls, 7 and 28 days after cavity preparation. Our ELISA data indicate that exposing the tooth pulp for 28 days, with confirmed inflammation, leads to a significant upregulation of BDNF in the TG ipsilateral to the affected teeth. Double-immunohistochemistry with antibodies against BDNF combined with one of nociceptor markers, CGRP or TRPV1, revealed that BDNF is significantly upregulated in TRPV1-immunoreactive (IR) neurons in both rats and mice, and CGRP-IR neurons in mice, but not rats. Overall, the inflammation-induced upregulation of

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BDNF is stronger in mice compared to rats. Thus, mouse TG provides a suitable model to study molecular mechanisms of inflammation-dependent regulation of BDNF expression *in vivo*.

Keywords

CGRP; ELISA; Mouse; Nociceptor; Rat; TRPV1

The trigeminal system, with first-order neurons in the trigeminal ganglion (TG), transmits sensory information from the majority of craniofacial tissues, including the meninges, temporomandibular joint and teeth (Shankland, 2000). Several common chronic pain conditions, such as migraines, chronic recurrent headaches, temporomandibular disorders and trigeminal neuralgias are mediated by trigeminal nociceptive pathways (Pietrobon and Striessnig, 2003; Scrivani et al., 2008; Prasad and Galetta, 2009). Yet, the molecular mechanisms of trigeminal nociceptive transmission and trigeminal pain are not fully understood.

The neurotrophin brain-derived neurotrophic factor (BDNF) is an established modulator of nociceptive signaling at the spinal (Mendell et al., 1999; Thompson et al., 1999; Mendell et al., 2001; Pezet et al., 2002; Malcangio and Lessman, 2003; Pezet and McMahon, 2006; Ren and Dubner, 2007; Merighi et al., 2008), and supraspinal (Guo et al., 2006; Ren and Dubner, 2007) levels. Previous studies from our and other laboratories show that BDNF is expressed by a large subpopulation of neonatal and adult rat TG neurons (Buldyrev et al., 2006; Ichikawa et al., 2006; Balkowiec and Balkowiec-Iskra, 2010). BDNF is localized to dense-core vesicles in axon terminals in the trigeminal *subnucleus caudalis* of the spinal trigeminal nucleus, the primary central target of trigeminal nociceptors (Buldyrev et al., 2006). Our studies indicate that BDNF is released from TG neurons by neuronal activity and that the release is regulated by calcitonin gene-related peptide (CGRP; Buldyrev et al., 2006), which is a key player in mechanisms of chronic trigeminal pain (Pietrobon and Striessnig, 2003). In addition, second-order neurons in trigeminal sensory nuclei of the brainstem express the high-affinity BDNF receptor, TrkB (Jacobs and Miller, 1999; King et al., 1999). Together, these findings raise the intriguing possibility that BDNF is involved in transmission of trigeminal pain at the level of primary sensory neurons and first-order synapses in the brainstem.

Trigeminal pain is frequently associated with peripheral inflammation. Moreover, peripheral inflammation enhances BDNF signaling in central pain modulatory circuitry (Guo et al., 2006; Ren and Dubner, 2007). Yet, the effects of an inflammatory process on regulation of BDNF availability in trigeminal pathways have not been examined. Therefore, we sought to establish an *in vivo* model of peripheral inflammation in the trigeminal system to study inflammation-dependent regulation of BDNF expression in trigeminal nociceptors.

Among the many populations of trigeminal afferents, those innervating the tooth pulp provide a particularly good model for studying trigeminal nociception because they represent a highly enriched, or possibly even pure, population of nociceptors (Eckert et al., 1997). In addition, peripheral endings of tooth pulp afferents are easily accessible compared to many other trigeminal afferents, thus making experimental manipulations, such as induction of inflammation, relatively straightforward. Since tooth pulp inflammation constitutes a common clinical problem, the model closely mimics the clinical setting.

Based on the evidence presented above, we chose tooth pulp inflammation evoked by exposing the pulp of first and second maxillary molars in 4-week-old rodents (Kakehashi et al., 1965; Byers and Närhi, 1999), as a model to study inflammation-induced changes in BDNF expression *in vivo*. We directly compared the rat model, which is commonly used in studies of trigeminal sensory transmission, with the mouse model, because of its tremendous potential

for studies of molecular mechanisms of trigeminal pain using genetically-engineered mice. We hypothesized that tooth pulp inflammation regulates BDNF expression in rat and mouse TG nociceptors *in vivo*. Portions of this work have previously been published in abstract form (Tarsa et al., 2007).

EXPERIMENTAL PROCEDURES

Animals and induction of tooth pulp inflammation

Juvenile (4-week-old) Sprague Dawley rats and C57BL/6J mice of both sexes (Charles River Laboratories, Wilmington, MA) were used in these studies. All animals were deeply anesthetized with Ketamine (50 mg/kg) / Xylazine (5 mg/kg) cocktail injected subcutaneously, weighed, and placed in a custom-made restraint system under Olympus SZ40 zoom stereomicroscope. Cavities were prepared in the right side maxillary first and second molars and remained unrestored, open to oral microflora for 7 and 28 days. The contralateral (left) side was intact and served as control. Additional control included animals that have been anesthetized and placed in the restraining system, but with unprepared teeth (sham-operated). All procedures were approved by the Institutional Animal Care and Use Committee of the Oregon Health and Science University, and conformed to the *Policies on the Use of Animals and Humans in Neuroscience Research* approved by the Society for Neuroscience.

Experimental groups and overview of procedures

Seven or 28 days after cavity preparation, animals were divided into two groups: 1) for quantitative evaluation of BDNF levels in the trigeminal ganglion (TG) by enzyme-linked immunosorbent assay (ELISA), and 2) for analysis of BDNF co-localization with nociceptor markers, CGRP and TRPV1, in TG neurons using immunohistochemistry. All animals were euthanized with Euthasol[®], weighed, and trigeminal ganglia were dissected either immediately (for ELISA) or following transcardial perfusion with 2% paraformaldehyde in 0.1 M phosphate buffer (for immunohistochemistry). In addition, the maxillary jaw segments of both sides, containing first and second molars, were removed from each animal and processed for histochemical staining. Only those animals in which tooth pulp inflammation was verified histologically were included in the analysis.

BDNF ELISA

Dissected trigeminal ganglia were immediately transferred to individual siliconized (Sigmacote[®]; Sigma), pre-weighed and pre-chilled 1.5-ml microcentrifuge tubes. The ganglia-containing tubes were weighed, and 100 μ l of pre-chilled lysis buffer (20 mM Tris buffer, pH 7.4, 137 mM NaCl, 1% Nonidet-P40, 10% glycerol, 1 mM phenylmethanesulfonyl fluoride (PMSF), 0.5 mM sodium vanadate, 10 μ M aprotinin, 10 μ M actinonin, and 100 μ M leupeptin) was added to each tube, followed by mechanical grinding of the ganglia with Kontes[®] Pellet Pestle[®] (Kimble-Chase, Vineland, NJ). Next, 400 μ l of Block & Sample buffer (BDNF E_{max}[™] ImmunoAssay System, Promega) was added, and samples (the total volume of 500 μ l) were sonicated on ice using a microprobe sonicator (3 $\times$ 3.0 W, 5 sec each; Sonicator 3000, Misonix, Inc., Farmingdale, NY). The resulting crude lysate of each ganglion was transferred to an anti-BDNF-coated 96-well ELISA plate (100 μ l per well; 3 wells per sample). BDNF ELISA was performed according to the manufacturer's protocol (BDNF E_{max}[™] ImmunoAssay System, Promega). BDNF levels were calculated from the standard curve prepared for each plate, using SOFTmax PRO[®] vs. 4.3 software (Molecular Devices). The standard curves were linear within the range used (0–500 pg/ml) and the quantities of BDNF in experimental samples were always within the linear range of the standard curve.

Immunohistochemistry of trigeminal ganglia

Trigeminal ganglia were removed from perfused animals, post-fixed with 2% paraformaldehyde in 0.1 M phosphate buffer for 1 hour, and processed as previously described by our laboratory (Buldyrev et al., 2006; Martin et al., 2009). Longitudinal sections (10- μ m thickness) were cut with a cryostat, collected in a series of 3 or 4, and two consecutive series were routinely processed: one for BDNF and CGRP, and the other for BDNF and TRPV1 (VR1, N-terminus), resulting in alternate sections being stained for each marker, i.e. CGRP and TRPV1. Double-immunohistochemistry was performed as previously described (Buldyrev et al., 2006; Martin et al., 2009) with chicken polyclonal anti-BDNF (1:50; Promega), and either rabbit polyclonal anti-CGRP (1:2000; Calbiochem) or rabbit polyclonal anti-TRPV1 (1:500; Neuromics, Edina, MN). BDNF was visualized using Cy3-conjugated goat anti-chicken IgG (1:200, Jackson ImmunoResearch), whereas CGRP and TRPV1 were visualized with Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:200, Molecular Probes).

Specificity of antibodies—The chicken polyclonal anti-BDNF was precleared with cerebellar slices, as previously described by our laboratory (Martin et al., 2009), in order to minimize any non-specific staining. Specificity of the precleared antibody was demonstrated by pre-absorption with BDNF (Martin et al., 2009). Specificity of other primary antibodies has been demonstrated by other laboratories (anti-CGRP, Franco-Cereceda et al., 1987; anti-TRPV1, Bennett et al., 2003). TG specimens stained according to the methods described above, but in the absence of primary antibodies, were completely devoid of staining for all secondary antibodies used (data not shown).

Microscopy and image analysis

Trigeminal ganglion sections were imaged with an Olympus IX-71 inverted microscope (Olympus America Inc., Center Valley, PA). To ensure the most comprehensive and accurate outcome, CGRP- and TRPV1-immunoreactive (IR) profiles, and the degree of BDNF-IR and CGRP-IR, or BDNF-IR and TRPV1-IR co-localization in each specimen, were analyzed independently by two investigators blinded to the group identity, and the results averaged. All CGRP-IR or TRPV1-IR profiles were selected first, blinded to BDNF content. Only cells with clearly visible nuclear profiles were included in the analysis. A cell was considered BDNF-, CGRP- or TRPV1-IR when the fluorescence intensity was clearly above the background. The background level was established independently by each investigator and used for all analyzed images.

Since the trigeminal ganglion is somatotopically organized, all CGRP-IR or all TRPV1-IR profiles were counted in two non-overlapping and distant fields of every section on a slide (one series), in order to sample functionally distinct regions of the ganglion in a systematic manner. Overall, one field was arbitrarily chosen in the mandibular division and the other in the ophtho-maxillary division. Every section in a series (i.e., every 3rd or 4th section of the intact ganglion, equally spaced, respectively, 30 μ m or 40 μ m apart from each other *in vivo*) was analyzed using a 400x magnification (40x objective and 10x eyepieces; the size of a single field, 0.238 mm²). Next, the number of BDNF-IR profiles within the CGRP-IR or TRPV1-IR population was counted in each analyzed field. For each ganglion, the total number of counted cells belonging to each of the four categories (CGRP-IR, CGRP-/BDNF-IR, TRPV1-IR, or TRPV1-/BDNF-IR) was added up, divided by the number of fields examined, and expressed as the cell density (number of cells per mm²).

For presentation in Figures 3A and 4A, images were captured with a Hamamatsu ORCA-ER CCD camera (Hamamatsu, Bridgewater, NJ) controlled by Olympus Microsuite software (vs. 5.0, Olympus America Inc). The original gray-scale images were pseudo-colored by first converting them to the RGB format using the Olympus Microsuite software.

Histological evaluation of tooth pulp inflammation

The maxillary jaw segments containing first and second molars were removed bilaterally en bloc, post-fixed in 10% neutral formalin for at least 3 days, and decalcified in a phosphate-buffered 10% EDTA solution at 4 °C for 2 weeks. Paraffin embedding, microtome sectioning and staining with hematoxylin and eosin were performed by Portland Tissue Lab (Portland, OR). Pulpal inflammation was evaluated with light microscopy by the investigator blinded to specimen identity. The presence of neutrophil infiltrations, a classic manifestation of an acute purulent inflammation, and abscesses, formed by neutrophils and necrotic tissue, were used as the criteria of pulpal inflammation. Images presented in Figure 1 were not processed in any way.

Statistical Analysis

Two-sample comparisons were analyzed with Student's *t*-test and multiple group data were analyzed by analysis of variance (ANOVA), followed by a *post hoc* comparison of means (Duncan's test). Data are expressed as mean $\pm$ standard error. $P < 0.05$ was considered significant.

RESULTS

Open cavity procedure can be performed on maxillary molars of rats and mice as young as 4 weeks of age, and leads to tooth pulp inflammation

We first sought to determine whether cavities can be prepared and tooth pulp inflammation evoked in juvenile rats and mice, shortly after the root formation. Our data indicate that rats and mice as young as 4 weeks of age can be successfully operated, including 100% success rates with long-term survival afterward. Moreover, we found characteristic features of an acute inflammatory process, such as infiltrations of neutrophils in the periapical region, as early as 7 days after the cavity preparation (Fig. 1A). Furthermore, indications of more severe, chronic inflammation, such as pulpal and periapical abscesses, were commonly seen 28 days after the cavity preparation (Fig. 1B). Out of 35 rats with open cavities prepared at 4 weeks of age, 14 (93.3%; 1 failure) and 15 (75.0%; 5 failures) showed histological signs of the inflammatory process in the tooth and/or surrounding tissues, respectively, 7 and 28 days after the cavity preparation. Out of 19 mice with open cavities prepared at 4 weeks of age, 6 (66.7%; 3 failures), 3 (75%; 1 failure), and 6 (100%, 0 failures) showed histological signs of inflammation, respectively, 7, 14, and 28 days after the cavity preparation. None of the right maxillary molars from sham-operated animals showed any histological evidence of inflammation (6 rats and 2 mice; Fig. 1C). Similarly, out of 12 (6 rats and 6 mice) maxillae containing molar teeth on the contralateral side (control), none showed any histological signs of an inflammatory process (Fig. 1D).

Previous studies indicate that a reduction in body weight can be used as an indirect measure of changes in feeding behavior that result from pain (Iadarola et al., 1988; Malcangio and Bowery, 1994), including tooth pain (Chudler and Byers, 2005). Therefore, in order to begin addressing the issue of behavioral relevance of our model, we have compared body weights of animals with active tooth pulp inflammation, as determined by the histological analysis, with sham-operated age-matched controls. Seven days after the cavity preparation, both rats and mice were characterized by a strong trend towards lower body weights (Rats, sham-operated: 114.5 ± 3.12 g, tooth pulp inflammation: 103.5 ± 3.13 g, $n=6$, $p=0.0661$; Mice, sham-operated: 21.0 ± 0.69 g, tooth pulp inflammation: 19.0 ± 0.61 g, $n=4$, $p=0.095$). The body weight differences reached statistical significance 4 weeks after the open cavity procedure in rats (sham-operated: 225.8 ± 0.58 g, tooth pulp inflammation: 209.3 ± 3.38 g, $n=6$, $p<0.05$).

Tooth pulp inflammation increases BDNF protein expression in trigeminal ganglia (TG)

We next examined levels of BDNF protein in TG from rats and mice 7 and 28 days after the open cavity preparation, using a highly sensitive enzyme-linked immunosorbent assay (ELISA) with BDNF-specific antibodies. Seven days after the cavity preparation, rat and mouse TG showed a trend towards increased BDNF levels in the ganglia ipsilateral to the tooth inflammation (right side) compared to the contralateral (left) ganglion, however, this difference was not statistically significant (Fig. 2 A, B, 7 days). By 28 days after the cavity preparation on the right side, the increases in BDNF levels in right TG compared to the left-side controls became unambiguous (Fig. 2 A, B, 28 days). Interestingly, both control and tooth pulp inflammation-induced BDNF protein levels, expressed per gram of tissue, were several-fold higher in mice compared to age-matched rats (Fig. 2 A vs. B). There were no differences in BDNF levels between right and left TG in sham-operated controls (872.42 ± 167.49 pg/g tissue in the right rat ganglion vs. 823.47 ± 174.37 pg/g tissue in the left ganglion).

TRPV1-immunoreactive subpopulation of TG neurons responds to tooth pulp inflammation with increased BDNF expression

The dramatic increase in the total BDNF content of the TG ipsilateral to the tooth pulp inflammation suggested that the upregulation of BDNF is more widespread than originally expected, and it cannot be accounted for solely by increases in BDNF expression within the small subset of cells innervating the two inflamed teeth. A vast majority of trigeminal nociceptors are small-diameter, unmyelinated C-type neurons (Lazarov et al., 2002). Therefore, to explore the functional characteristic of the TG population which increases BDNF expression in response to peripheral inflammation, we first focused on a commonly used marker of small, unmyelinated nociceptors, the vanilloid receptor TRPV1. In addition, TRPV1 has been shown to regulate neuropeptide secretion from TG neurons under inflammatory conditions (Price et al., 2005). We used a TRPV1/BDNF double-immunohistochemistry approach, as in our previous studies (Martin et al., 2009).

We found that in intact animals, over 40% of all TRPV1-immunoreactive (IR) TG neurons also express BDNF in rats, whereas this percentage is markedly lower (less than 20%) in mice (Fig. 3A). In animals with tooth pulp inflammation on the right side, the mean density of BDNF/TRPV1-IR cells was significantly elevated in the right TG already 7 days after the cavity preparation. The magnitude of the increase was not changed by the duration of the inflammatory process, but it was significantly larger in mice (2.3-fold and 2.2-fold increase, respectively, 7 and 28 days after the cavity preparation) compared to rats (1.4-fold and 1.3-fold increase, respectively, 7 and 28 days after the cavity preparation; Fig. 3B). When expressed as a fraction of all TRPV1-IR cells, the number of BDNF-IR cells increased from 42.3 ± 6.82 % in the contralateral ganglion to 49.9 ± 2.96 % in the ganglion ipsilateral to the inflammatory process in rats, and from 16.8 ± 5.94 % to 34.8 ± 5.24 % in mice, 7 days after the induction of inflammation. The additional 3 weeks of inflammation resulted in BDNF-IR cell increases from 48.8 ± 3.41 % to 52.6 ± 6.13 % in rats, and from 24.2 ± 2.46 % to 40.6 ± 3.04 % of all TRPV1-IR cells in mice (Fig. 3 B, C). A comparative analysis of the BDNF and TRPV1 double-immunoreactivity in the caudally located mandibular division and the ophthalmic-maxillary/bifurcation region, which is known to contain cell bodies of neurons innervating maxillary molars (Shellhammer et al., 1984; Barnett et al., 1995), revealed no significant differences in the inflammation-induced upregulation of BDNF/TRPV1 between these two regions in any of the ganglia examined ($p > 0.05$).

Consistent with previous studies demonstrating that peripheral inflammation increases TRPV1 expression (Amaya et al., 2004; Breese et al., 2005), there was a significant increase in the mean density of all TRPV1-IR cells, both 7 and 28 days after the inflammation was evoked in rats and mice (Fig. 3C). Therefore, the increases in the density of BDNF/TRPV1-IR cells could

potentially be accounted for by increases in the density of TRPV1-IR cells in rats (1.2-fold increase, both 7 and 28 days after the cavity preparation; Fig. 3C), suggesting a parallel increase in both BDNF and TRPV1 in response to peripheral inflammation. In mice, on the other hand, the increase in the mean density of TRPV1-IR cells (1.1-fold and 1.3-fold, respectively, 7 and 28 days after the cavity preparation; Fig. 3C) was significantly smaller than the increase in the density of BDNF-/TRPV1-IR cells. The latter result suggests that, in mice, the inflammation-induced increase in BDNF expression is stronger than the increase in TRPV1 expression.

Tooth pulp inflammation-induced upregulation of BDNF includes the calcitonin gene-related peptide (CGRP)-immunoreactive neurons in mouse, but not rat, TG

Many small- to medium-size TG nociceptors express CGRP, which plays a critical role in mechanisms of neuroinflammation and peripheral sensitization of nerve endings (Lazarov, 2002). Previous studies have demonstrated that CGRP expression and release in dorsal root ganglion and TG neurons are increased by peripheral inflammation (Donaldson et al., 1992; Nahin and Byers, 1994; Malcangio et al., 1997; Durham and Russo, 1999; Bulling et al., 2001; Xu and Hall, 2007). Also, we have previously shown that BDNF is expressed by a large proportion of CGRP-IR TG neurons in rats, and CGRP stimulates BDNF release from rat TG neuron cultures (Buldyrev et al., 2006). Therefore, we next examined the effects of tooth pulp inflammation on BDNF expression within the CGRP-IR subpopulation of TG neurons in rats and mice, using BDNF/CGRP double-immunohistochemistry, as in our previous studies (Buldyrev et al., 2006).

Our data indicate that, in intact animals, approximately 20% of all CGRP-IR TG neurons also express BDNF in both rats and mice (Fig. 4A, and Buldyrev et al., 2006, Fig. 1). In the present study, in rats, the mean density of TG neurons that express double BDNF/CGRP immunoreactivity was not affected by tooth pulp inflammation in any region of the ganglion examined, regardless of the duration of inflammation (Fig. 4B). Similarly, the percentage of BDNF-IR cells among CGRP-IR cells was not altered (7-day inflammation: 17.9 ± 1.03 % in left TG vs. 18.6 ± 4.30 % in right TG; 28-day inflammation: 19.0 ± 1.48 % in left TG vs. 19.6 ± 5.19 % in right TG, $n=3$). Unlike rats, the mean density of mouse TG neurons that showed double BDNF/CGRP immunoreactivity increased significantly 28, but not 7, days after the induction of inflammation (Fig. 4B). The overall number of CGRP-IR cells was not affected by the inflammation, either in rats or mice (Fig. 4C). Similar to BDNF/TRPV1 double-immunoreactivity, there were no significant differences in the magnitude of BDNF/CGRP upregulation between the mandibular and ophthalmic-maxillary division in any of the ganglia examined ($p>0.05$).

DISCUSSION

The present study shows that acute and chronic tooth pulp inflammation can be evoked in both rats and mice as early as advanced stages of molar tooth development, thus offering a new model for studies of the effects of inflammation on the developing trigeminal system. Our study also provides the first evidence that tooth pulp inflammation leads to an upregulation of BDNF protein in ipsilateral trigeminal ganglion (TG), an effect that is more widespread than can be accounted for by an exclusive activation of pulpal afferents from the affected teeth. The results of quantitative analysis of BDNF expression by ELISA were consistent with the data obtained from counts of BDNF-immunoreactive (IR) TG neurons on the side of inflammation, compared to the contralateral TG. The counts were performed in neurons specifically identified by double-immunohistochemistry with two markers of nociceptors, calcitonin gene-related peptide (CGRP) and the vanilloid receptor TRPV1. While in the rat model, only the TRPV1-IR subpopulation showed a statistically significant, though moderate, upregulation of BDNF, both CGRP-IR and TRPV1-IR subpopulations were characterized by a significant BDNF

upregulation in the mouse model. In fact, both ELISA and immunohistochemistry data indicate that the effects of tooth pulp inflammation on the regulation of BDNF expression in TG are significantly stronger in mice compared to age-matched rats. To our knowledge, the current study is the first to find the difference between the two animal models and examine BDNF colocalization with CGRP and TRPV1 in the mouse TG. In addition, this is one of the few reports to date using the mouse model of tooth pulp inflammation for studies of trigeminal sensory pathways.

The importance of understanding the mechanisms of nociceptive transmission in trigeminal pathways is underscored by the fact that trigeminal pain, including temporomandibular disorders, trigeminal neuralgia, migraine headaches, and toothache, constitute common sources of chronic craniofacial pain (Henry and Hargreaves, 2007; Kopp, 2001; Sessle, 1999, 2000; Tenenbaum et al., 2001; Woda and Pionchon, 2000). Several mechanisms have been implicated in the pathophysiology of trigeminal pain, including peripheral sensitization of nerve endings (Dubner, 2004; Olesen et al., 2009; Sessle, 1999; Woda and Pionchon, 2000), central neuroplasticity and hyperexcitability (Iwata et al., 1999; Ren and Dubner, 1999; Sessle, 1999; Zhou et al., 1999; Ren and Dubner, 2007) and release of inflammatory mediators (Kopp et al., 2001). Although the exact mechanisms of orofacial pain conditions are not fully understood, it is unquestionable that the nervous system plays a pivotal role, and that the vast majority of trigeminal pain conditions are associated with an inflammatory process. Yet, the availability of animal models of trigeminal nociception, including inflammatory pain, remains limited, and the current study addresses this issue.

BDNF is involved in activity-dependent modifications of synaptic transmission and plasticity in sensory systems (Balkowiec et al., 2000; Mendell et al., 1999, 2001; Pezet et al., 2002). Previous studies from our laboratory provide several lines of evidence that BDNF is a mediator of synaptic transmission and plasticity at first-order synapses in the trigeminal nociceptive pathways (Buldyrev et al., 2006; Balkowiec and Balkowiec-Iskra, 2010). Moreover, studies from other laboratories indicate that BDNF is expressed in human TG (Quartu et al., 1997), rat tooth pulp afferents (Ichikawa et al., 2006) and that it can be induced in TG neurons (Pan et al., 2000). In addition, the high-affinity receptor for BDNF, trkB, is expressed in TG neurons and its expression is regulated by tooth injury (Behnia et al., 2003; Foster et al., 1995; Quartu et al., 1996; Wheeler et al., 1998). For these reasons, we focused the current study on examining the effects of tooth pulp inflammation on BDNF expression in first-order sensory neurons in the trigeminal system.

Our data are consistent with previous studies, in which increased BDNF mRNA and BDNF immunoreactivity were observed in rat dorsal root ganglion neurons and their central terminations in the spinal cord, following paw inflammation evoked by an intraplantar injection of Freund's adjuvant (Cho et al., 1997a,b). We found that the increase in BDNF protein expression in response to pulpal inflammation was widespread and most likely not limited to the subpopulation of TG neurons that innervated the inflamed tooth pulps. In support, we observed increases in BDNF expression within the population immunoreactive for TRPV1, a marker of small unmyelinated afferents, whereas tooth pulp is innervated primarily by myelinated afferents with larger cell body diameters (Lazarov, 2002; Ichikawa et al., 2006; Paik et al., 2009). A similar effect was previously reported regarding CGRP and substance P expression in the rat TG following inflammation of the temporomandibular joint, including an increased neuropeptide expression in the contralateral ganglion (Hutchins et al., 2000). The result suggests an indirect effect, through a diffusible factor that is induced in response to the inflammation and/or behavioral stress associated with the inflammatory pain. One potential group of diffusible factors that likely play a role in regulation of BDNF expression *in vivo* are pro-inflammatory cytokines. Recent studies from our laboratory indicate that BDNF mRNA and protein expression are dramatically upregulated in TG neurons *in vitro* by pro-

inflammatory cytokines, including tumor necrosis factor- α (Bałkowiec-Iskra and Balkowiec, 2009; Balkowiec and Bałkowiec-Iskra, 2010), shown to play a role in trigeminal inflammation. However, the specific mechanism of the more generalized upregulation of BDNF in response to tooth pulp inflammation remains to be elucidated.

Our study indicates that basal levels of, as well as inflammation-induced changes in, BDNF expression are larger in mice compared to age-matched rats. Indeed, previous comparative studies of various tissues, including the brain, show that specific tissues are characterized by several-fold higher BDNF levels in mice, compared to rats of the same developmental stage (Kato-Semba et al., 1998). Moreover, other data provide the molecular basis for the tissue-specific regulation of the BDNF gene, and indicate its large diversity with a multitude of species- and stimulus-specific regulatory mechanisms (Liu et al., 2006; Aid et al., 2007). Although those could potentially explain the differences in the magnitude of inflammation-induced upregulation of BDNF expression between TG neurons of rats and mice, more studies are needed to specifically address this issue.

Data obtained by other investigators in dorsal root ganglion neurons of spinal nociceptive pathways indicate that CGRP is upregulated by peripheral inflammation in several experimental models (Donaldson et al., 1992; Nahin and Byers, 1994; Malcangio et al., 1997; Bulling et al., 2001; Xu and Hall, 2007). Our current data from the trigeminal system show that, while tooth pulp inflammation significantly increases the number of BDNF-IR and TRPV1-IR neurons, it does not affect the number of CGRP-IR neurons. In support of the possibility that CGRP is, indeed, unaffected by tooth pulp inflammation, it has previously been demonstrated in a rat model of complete Freund adjuvant-induced single joint inflammation that CGRP can be unaffected by the inflammation while another sensory peptide, substance P, is strongly upregulated (Malcangio and Bowery, 1996). Another potential explanation would be the possibility that CGRP expression increased only in cells that already expressed CGRP and, consequently, the number of CGRP-IR cells did not increase. It is also plausible that CGRP, unlike BDNF and TRPV1, was upregulated exclusively in the small population of TG neurons innervating the inflamed teeth and, for that reason, the upregulation was missed during the analysis of CGRP-IR cell density in the general population of unidentified trigeminal ganglion neurons. The results of our ELISA analysis of total BDNF content that suggested a widespread upregulation of this neurotrophin, combined with the anticipation of serious challenges of neuronal tracing in young mice, prompted us to focus on addressing the issue of the potential upregulation of BDNF in neurons not directly connected with inflamed tissues, and perform a comprehensive analysis of the entire trigeminal ganglion. Future studies will examine inflammation-induced changes in neurochemical properties of tooth pulp afferents specifically identified by retrograde tracing.

We chose tooth pulp inflammation as a model for studies of inflammation-induced changes in BDNF expression in the context of trigeminal inflammatory pain because teeth receive predominantly nociceptive innervation. We have determined that neurons expressing TRPV1, a marker of nociceptors, upregulate BDNF. This finding offers an indirect evidence that nociceptor properties are altered in our model. However, the present study does not provide specific behavioral data to fully support the notion that the employed model of tooth pulp inflammation is a pain model, and its behavioral relevance still needs to be determined.

Together, these data shed light on the activity-dependent regulation of BDNF availability in response to inflammation in the trigeminal system. To our knowledge, this is the first study to use the mouse tooth pulp inflammation as a model for studies of this kind. Our results will likely facilitate the utilization of transgenic mice and, therefore, represent a novel approach towards understanding molecular mechanisms of trigeminal sensory transmission.

LIST OF ABBREVIATIONS

BDNF	brain-derived neurotrophic factor
CGRP	calcitonin gene-related peptide
ELISA	enzyme-linked immunosorbent assay
IR	immunoreactive
TG	trigeminal ganglion
TRPV1	transient receptor potential vanilloid type 1 channel

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Figure 1.

Photomicrographs of hematoxylin/eosin-stained sagittal sections through the first right (A–C) and left (D) maxillary molar of a mouse (A, C) and rat (B, D). A cavity was prepared on the right side at 4 weeks of age, and was left open to oral microflora for 7 days (A) and 28 days (B). The periapical region shows infiltrates of neutrophils and pulp abscesses (A, B; insets), which are absent in a sham operated animal (C) and on the intact left side (D). Scale bars, 500 μm.

Figure 2.

Levels of BDNF protein (per gram of tissue) in left (Control) and right (Inflammation) trigeminal ganglia, seven and 28 days after the open cavity preparation in first and second maxillary molars on the right side in (A) rats (7 days, n=8 rats; 28 days, n=11 rats), and (B) mice (7 days, n=3 mice; 28 days, n=4 mice); * $P < 0.05$, ** $P < 0.01$.

A. **B.** **C.** **Figure 3.**

(A) Micrographs of a single 10- μ m section of the trigeminal ganglion from a 5-week-old rat double-immunostained for BDNF (α BDNF) and TRPV1 (α TRPV1). An overlay image (Overlay) shows that many TRPV1-immunoreactive cells also express BDNF (arrows). Scale bar, 100 μ m. (B) Mean density of BDNF/TRPV1 double-immunoreactive (IR) cells in left (Control) and right (Inflammation) trigeminal ganglia from 5-week-old rats (left panel) and mice (right panel) with cavities prepared in right-side first and second molars at 4-weeks of age. (C) Mean density of all (i.e., BDNF-IR and BDNF-non-IR) TRPV1-IR cells counted in the same microscope fields as BDNF/TRPV1-IR cells presented in panel B. Numbers of analyzed cells: Rat, 7 days: Control, 1789 TRPV1-IR and 757 BDNF/TRPV1-IR,

Inflammation, 2000 TRPV1-IR and 998 BDNF/TRPV1-IR (N=2 rats); 28 days: Control, 1211 TRPV1-IR and 591 BDNF/TRPV1-IR, Inflammation, 2332 TRPV1-IR and 1226 BDNF/TRPV1-IR (N=3 rats). Mouse, 7 days: Control, 438 TRPV1-IR and 139 BDNF/TRPV1-IR, Inflammation, 1967 TRPV1-IR and 758 BDNF/TRPV1-IR; 28 days: Control, 4792 TRPV1-IR and 1160 BDNF/TRPV1-IR, Inflammation, 6508 TRPV1-IR and 2644 BDNF/TRPV1-IR. (N=3 mice); * $P<0.05$; *** $P<0.001$.

Figure 4.

(A) Micrographs of a single 10- μ m section of the trigeminal ganglion from a 5-week-old mouse double-immunostained for BDNF (α BDNF) and CGRP (α CGRP). An overlay image (Overlay) shows that some CGRP-positive cells also show BDNF immunoreactivity (arrows). Scale bar, 50 μ m. (B) Mean density of BDNF/CGRP double-immunoreactive (IR) cells in left (Control) and right (Inflammation) trigeminal ganglia from 5-week-old rats (left panel) and mice (right panel) with cavities prepared in right-side first and second molars at 4-weeks of age. (C) Mean density of all (i.e., BDNF-IR and BDNF-non-IR) CGRP-IR cells counted in the same fields as BDNF/CGRP-IR cells presented in panel B. The analysis of BDNF/CGRP-IR cells was performed on alternate sections of the ganglia used for the analysis of BDNF/TRPV1-IR cells

presented in Figure 3. Numbers of analyzed cells: Rat, 7 days: Control, 2450 CGRP-IR and 440 BDNF/CGRP-IR, Inflammation, 2550 CGRP-IR and 474 BDNF/CGRP-IR (N=2 rats); 28 days: Control, 2642 CGRP-IR and 628 BDNF/CGRP-IR, Inflammation, 3344 CGRP-IR and 654 BDNF/CGRP-IR (N=3 rats). Mouse, 7 days: Control, 1490 CGRP-IR and 548 BDNF/CGRP-IR, Inflammation, 1262 CGRP-IR and 442 BDNF/CGRP-IR; 28 days: Control, 3079 CGRP-IR and 699 BDNF/CGRP-IR, Inflammation, 2967 CGRP-IR and 838 BDNF/CGRP-IR. (N=3 mice); *** $P < 0.001$; ns, non-significant.