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TUMOR NECROSIS FACTOR- α INCREASES BDNF EXPRESSION IN TRIGEMINAL GANGLION NEURONS IN AN ACTIVITY- DEPENDENT MANNER

Ewa Bałkowiec-Iskra^{1,2}, Anke Vermehren-Schmaedick¹, and Agnieszka Bałkowiec^{1,3}
Dr. Linda S. Sorkin

¹Department of Integrative Biosciences, Oregon Health & Science University School of Dentistry, Portland, Oregon ²Department of Experimental & Clinical Pharmacology, Medical University of Warsaw, Warsaw, Poland ³Department of Physiology & Pharmacology, Oregon Health & Science University School of Medicine, Portland, Oregon.

Abstract

Many chronic trigeminal pain conditions, such as migraine or temporo-mandibular disorders, are associated with inflammation within peripheral endings of trigeminal ganglion (TG) sensory neurons. A critical role in mechanisms of neuroinflammation is attributed to proinflammatory cytokines, such as interleukin-1 β and tumor necrosis factor- α (TNF α) that also contribute to mechanisms of persistent neuropathic pain resulting from nerve injury. However, the mechanisms of cytokine-mediated synaptic plasticity and nociceptor sensitization are not completely understood. In the present study, we examined the effects of TNF α on neuronal expression of brain-derived neurotrophic factor (BDNF), whose role in synaptic plasticity and sensitization of nociceptive pathways is well documented. We show that 4- and 24-hr treatment with TNF α increases BDNF mRNA and protein, respectively, in neuron-enriched dissociated cultures of rat TG. TNF α increases the phosphorylated form of the cyclic adenosine monophosphate-responsive element binding protein (CREB), a transcription factor involved in regulation of BDNF expression in neurons, and activates transcription of BDNF exon IV (former exon III) and, to a lesser extent, exon VI (former exon IV), but not exon I. TNF α -mediated increase in BDNF expression was accompanied by increase in calcitonin gene-related peptide (CGRP), which is consistent with

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Corresponding author: Dr. Agnieszka Bałkowiec, Department of Integrative Biosciences, Oregon Health and Science University School of Dentistry, 611 S.W. Campus Drive, Portland, OR 97239; Phone: (503) 418-0190; Fax: (503) 494-8554; balkowie@ohsu.edu. All experimental protocols are available in a detailed, step-by-step format upon request from Dr. Ewa Bałkowiec-Iskra (ebalkowiec@wum.edu.pl).

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Research Highlights:

1. BDNF mRNA and protein are upregulated in trigeminal ganglion neurons by TNF- α
2. TNF- α upregulates BDNF expression in a promoter-selective manner
3. TNF- α upregulation of BDNF expression is potentiated by neuronal activity and cAMP
4. TNF- α upregulation of BDNF expression depends on sodium channels and p38-MAPK
5. These data have implications for BDNF-dependent sensory plasticity

previously published studies, and indicates that both peptides are similarly regulated in TG neurons by inflammatory mediators. The effect of TNF α on BDNF expression is dependent on sodium influx through TTX-sensitive channels and on p38-mitogen-activated protein kinase. Moreover, electrical stimulation and forskolin, known to increase intracellular cAMP, potentiate the TNF α -mediated upregulation of BDNF expression. This study provides new evidence for a direct action of proinflammatory cytokines on TG primary sensory neurons, and reveals a mechanism through which TNF α stimulates *de novo* synthesis of BDNF in these neurons. Thus, TNF α should be considered in mechanisms of BDNF-dependent neuronal plasticity.

Keywords

Activity; BDNF; Inflammation; p38-MAPK; TNF α ; Trigeminal

Recent studies provide several lines of evidence for a cross-talk between the nervous and immune systems. Many disorders of the central and peripheral nervous system, including Alzheimer's and Parkinson's disease, and a variety of pain conditions, are accompanied by inflammation of the neural tissue which is affected by the disease process ('neuroinflammation'; McAllister and van de Water, 2009). Moreover, there is increasing evidence that the immune system contributes to normal neuronal development and plasticity (Vitkovic et al., 2000; Boulanger, 2009; Carpentier and Palmer, 2009). Thus, identifying the mechanisms that underlie neuro-immune interactions could become fundamental to understanding how the nervous system functions under physiological conditions and what goes awry in the disorders whose pathogenesis involves an immunological component.

Cytokines, first discovered as signaling molecules within the immune system, are also produced by the nervous system. Two prototypic proinflammatory cytokines, interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF α), are released from glia and regulate development and plasticity of neuronal circuits (Deverman and Patterson, 2009), including nociceptive pathways (Schäfers and Sorkin, 2008; McMahon and Malcangio, 2009; Ren and Torres, 2009). Cytokines and other inflammatory mediators have been shown to alter ion currents in the peripheral endings of nociceptors (Binshtok et al., 2008), including trigeminal neurons (Flake and Gold, 2005; Vaughn and Gold, 2010), leading to increased excitability and sensitization. However, the role of cytokines in inflammatory and neuropathic pain is not completely understood (Schäfers and Sorkin, 2008; Basbaum et al., 2009; Costigan et al., 2009; Latremoliere and Woolf, 2009).

The neurotrophin brain-derived neurotrophic factor (BDNF) is a mediator of plasticity at central synapses (Huang and Reichardt, 2001; Poo, 2001; Cohen and Greenberg, 2008), and modulator of nociceptive signaling (Pezet et al., 2002; Malcangio and Lessman, 2003; Merighi et al., 2008; Latremoliere and Woolf, 2009). Previous studies by our group indicate that BDNF is a candidate mediator of plasticity at first-order synapses in trigeminal nociceptive pathways (Buldyrev et al., 2006), with implications for pathophysiology of migraine and other primary headaches (Latremoliere and Woolf, 2009). Our recent studies show that inflammation within peripheral endings of trigeminal nociceptors dramatically upregulates BDNF in trigeminal ganglion (TG) neurons *in vivo* (Tarsa et al., 2010). However, the cellular mechanisms underlying the inflammation-induced BDNF upregulation remain unknown. Notably, several pain conditions, including migraine, are accompanied by inflammation within endings of TG neurons.

The goal of the present study was to examine the effects of the proinflammatory cytokine TNF α on BDNF expression, using sensory neurons isolated from the rat TG as a model. Our data indicate that TNF α increases BDNF mRNA and protein expression in TG neurons in a

promoter-selective manner. The effect of TNF α depends on TTX-sensitive sodium currents and the p38-MAPK pathway, and is potentiated by neuronal activity and intracellular cAMP. Our findings identify a novel mechanism of regulation of BDNF expression in TG neurons by TNF α . Portions of this work have previously been published in abstract form (Balkowiec-Iskra and Balkowiec, 2009).

EXPERIMENTAL PROCEDURES

Animals and preparation of TG cultures

Postnatal day 1-3 Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) were used for this study. 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. Neuron-enriched TG cultures were prepared as previously described by our laboratory (Buldyrev et al., 2006; Tarsa and Balkowiec, 2009), and grown in Neurobasal-A medium (Invitrogen, Carlsbad, CA) supplemented with B-27 (Invitrogen), 0.05 mg/ml penicillin- 0.05 mg/ml streptomycin-0.1 mg/ml neomycin (Invitrogen), 0.5 mM L-glutamine (Invitrogen), and 2.5% fetal bovine serum (HyClone, Logan, UT) for 4-6 days in 48-well tissue culture-treated plates (Nalge Nunc Int., Naperville, IL) pre-coated with poly-D-lysine (0.1 mg/ml; Sigma, St. Louis, MO) and laminin (0.4 μ g/ml; Sigma; for quantitative RT-PCR and ELISA), or on poly-D-lysine/laminin-coated glass coverslips (for immunocytochemistry). The medium was replaced with fresh medium every 2-3 days. In some experiments, the medium was supplemented with the mitotic inhibitor cytosine β -D-arabinofuranoside (AraC; 5 μ M, Sigma) approximately 24 hrs after cell plating to prevent non-neuronal cell proliferation. Since the results of experiments using AraC-treated cultures were not significantly different from cultures not treated with AraC, the data were pooled.

Electrical field stimulation of TG neurons

TG neurons were stimulated as previously described (Buldyrev et al., 2006), except that pairs of platinum electrodes (0.25 mm wire diameter) were fitted into 48-well plates, the pulse duration was 0.2 ms, and the current amplitude was 100 mA/well. All non-stimulated control wells were fitted with electrodes which were not connected to the stimulator.

Pharmacological treatment

Cultures were treated with tumor necrosis factor- α (TNF α) for 4 hrs (for BDNF mRNA by quantitative RT-PCR) or 24 hrs (for BDNF, CGRP and NGF protein by ELISA). Other drugs were applied 30 min prior to the electrical stimulation or cytokine treatment, and remained present until the end of culture. Each drug (or drug combination) and each vehicle (or vehicle combination) were added to both stimulated and non-stimulated cultures. Stock solutions of TNF α (100 μ g/ml in phosphate-buffered saline [PBS]; R&D Systems, Minneapolis, MN), tetrodotoxin (TTX; 3 mM in citrate buffer, pH=4.8; Tocris Bioscience, Ellisville, MO), 3-Isobutyl-1-methylxanthine (IBMX; 25 mM in ethanol; Sigma), and SB 203580 hydrochloride (10 mM in water; Tocris Bioscience) were stored at -80°C; forskolin stock (100 mM in ethanol; Sigma) was prepared fresh just before use.

Immuno- and DAPI-staining of TG cultures, and image analysis

Cultures were fixed with 4% (for NGF staining) or 2% (for BDNF staining) paraformaldehyde in 0.1 M sodium phosphate buffer, pH=7.4, for 30 min, and stained according to our previously published protocols (Buldyrev et al., 2006; Martin et al., 2009). The following primary and secondary antibody pairs were used: i) chicken anti-BDNF (1:50; Promega, Madison, WI) with either goat anti-chicken-biotinylated IgG (1:200; Vector

Laboratories, Burlingame, CA) or donkey anti-chicken IgG-Cy3 (1:200; Jackson Immunoresearch, West Grove, PA), ii) rabbit anti-NGF (1:1000; Millipore, Temecula, CA) with goat anti-rabbit IgG-Alexa 488 (1:200; Invitrogen), iii) mouse anti-Neurofilament 68 and 160 (1:100; Sigma) with goat anti-mouse IgG-Cy3 (1:200; Jackson Immunoresearch), and iv) rabbit anti-phospho-CREB (1:1000; Millipore) with goat anti-rabbit IgG-Alexa 488 (1:200; Invitrogen). Following secondary antibody application, the DNA stain 4',6-diamidino-2-phenylindole (DAPI; 300 nM in PBS, Invitrogen) was applied to some coverslips for 10 min at room temperature to label all cell nuclei. Control cultures, in which primary antibodies were omitted, were completely devoid of staining.

Stained TG cultures were imaged as recently described (Martin et al., 2009; Tarsa and Balkowiec, 2009). To determine the 'neuron to non-neuronal cell' ratio, the total number of cells with DAPI-positive nuclei and the total number of neurofilament-immunoreactive cells (putative neurons) were counted in at least 10 randomly chosen fields per coverslip (200x total magnification), in 6 independent vehicle-treated cultures. To determine the percentage of NGF-immunoreactive cells, all NGF- and all neurofilament-immunoreactive cells were counted in 12 randomly chosen fields per coverslip (200x total magnification), in 6 vehicle-treated and 6 TNF α -treated cultures.

Measurement of BDNF and CGRP cell content, and NGF release

At the end of treatment, the culture medium was collected from each well and replaced with 75 μ l of pre-chilled lysis buffer (Hsieh et al., 2010), followed by rinsing with 150 μ l of pre-chilled Block & Sample buffer (1x; BDNF E_{max}TM ImmunoAssay System, Promega). The content of two identically treated culture wells (the total volume of 450 μ l) was sonicated as recently described (Hsieh et al., 2010). BDNF and CGRP ELISAs were performed using BDNF E_{max}TM ImmunoAssay System (Promega), and Rat CGRP EIA (SPI-BIO; Montigny-le Bretonneux, France), respectively, according to the manufacturers' protocols. In some experiments, the medium collected from each culture well was immediately processed for detection of NGF by ELISA, using NGF E_{max}TM ImmunoAssay System (Promega) according to the manufacturer's instructions. BDNF, CGRP, and NGF levels were calculated from the standard curves prepared for each plate, using SOFTmax PRO® vs. 4.3 software (Molecular Devices, Sunnyvale, CA). The standard curves were linear within the range used (0-500 pg/ml for BDNF and CGRP; 0-250 pg/ml for NGF) and the quantities of BDNF, CGRP, and NGF in experimental samples were always within the linear range of the standard curve. The specificity of the BDNF ELISA has been confirmed by our laboratory using BDNF siRNA approach (Hsieh et al., 2010).

RNA extraction and quantitative RT-PCR

Quantitative RT-PCR was performed as recently described by our laboratory (Hsieh et al., 2010). Briefly, total RNA was extracted using TRIzol (Invitrogen), followed by re-suspension in 10 mM Tris pH 8.5. To make cDNA, 1 μ g of total RNA was reverse transcribed (20 μ l) using Tetro cDNA synthesis kit (Bioline, Taunton, MA) following the manufacturer's instructions. Quantitative PCR was performed in triplicates following the MIQE guidelines (Bustin et al., 2009) using the MX3000P real time PCR system (Stratagene; Cedar Creek, TX) in a final volume of 10 μ l using Sensimix Plus SYBR master mix (Quantace, Taunton, MA), 2 μ l of cDNA (diluted 1:4), and 3 μ M of each forward and reverse primers. All primer sequences were designed using Primer3 online software (<http://frodo.wi.mit.edu/primer3/>) and synthesized by Integrated DNA Technologies (Coralville, IA), for sequences see below. To check for primer specificity, DNA products were amplified by PCR using Taq polymerase (Invitrogen, Carlsbad, CA). Single PCR products were gel-purified using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA), sequenced (OHSU DNA sequencing facilities) and quantified spectrophotometrically.

Quantitative RT-PCR was performed with DNA standard dilutions ranging from 10pg to 10 ag. The real time amplification data were collected continuously using the software supplied with the MX3000P real time PCR system. Cq values for standard curves were between 10 and 30, with Cq values for samples falling within this range. RT-PCR efficiencies were all above 97%. The forward primers for BDNF are 5'-aaagccgaacttctcacatgat-3' (BDNF-I), 5'-ctcccccttttaactgaagagaa-3' (BDNF-IV), ctttggggcagacgagaaag-3' (BDNF-VI), with a common reverse primer (5'-attcacgctctccagagtcc-3'). The PCR products were 273, 280, and 275 bp, respectively. Data normalization was carried out against the endogenous reference gene transcript hypoxanthine-guanidine phosphoribotransferase (HPRT, forward: 5'-caggccagactttgttgat-3, reverse: 5'-ggctgcctacaggtcatag-3'; PCR product of 328 bp in size). Genbank accession numbers are EF125675.1 (BDNF-I), EF125679.1 (BDNF-IV/former BDNF-III), EF125680.1 (BDNF-VI/former BDNF-IV), and NM012583.2 (HPRT).

Calculations and statistical analysis

Data are expressed as mean $\pm$ standard error, and the sample size (n) represents the number of individual cultures. Samples were compared using Student's *t*-test or ANOVA followed by Duncan's multiple comparison procedure, and $P < 0.05$ was considered significant.

RESULTS

Tumor necrosis factor- α (TNF α) increases BDNF expression in trigeminal ganglion (TG) neurons *in vitro*

Proinflammatory cytokines have previously been shown to directly affect nociceptive neurons in a variety of ways (Schäfers and Sorkin, 2008; Basbaum et al., 2009; Costigan et al., 2009; Latremoliere and Woolf, 2009). However, it has not been established whether these influences include changes in expression of BDNF, a major player in mechanisms of synaptic plasticity.

To address this issue, we used our previously developed model of neuron-enriched rat TG cultures (Buldirev et al., 2006), in which the ratio of neurons to non-neuronal cells is approximately 1:3 (2.99 ± 0.24 non-neuronal cells identified by DAPI-stained nuclei per neuron identified by neurofilament immunoreactivity; $n = 6$ cultures not treated with AraC). We examined BDNF mRNA and protein expression, along with the cell content of calcitonin gene-related peptide (CGRP) as a control, in response to TNF α applied in the presence of different levels of neuronal activity and modulators of signaling pathways. We focused on TNF α because of its established role in mechanisms of pain hypersensitivity.

In the first set of experiments, we treated TG cultures with TNF α (1, 10 and 50 ng/ml) for 24 hrs in order to determine if this cytokine regulates BDNF protein synthesis. The range of TNF α concentrations was chosen based on previous studies of TNF α levels in the inflamed tooth pulp (Ataoglu et al., 2002; Pezelj-Ribaric et al., 2002; Bletsa et al., 2006), one of the major targets of trigeminal nociceptors and a source of BDNF upregulation by trigeminal neurons *in vivo* (Tarsa et al., 2010).

Treatment with 10 ng/ml of TNF α resulted in a highly significant, over 60% increase in BDNF cell content compared to vehicle (Fig. 1 A). A 10-fold lower concentration of TNF α , i.e. 1 ng/ml, did not have a significant effect on BDNF protein expression. On the other hand, the highest concentration of TNF α examined, i.e. 50 ng/ml, resulted in the increase in BDNF protein expression that was not significantly different from the effect of 10 ng/ml (Fig. 1A). Based on these results, in subsequent experiments of this study we used TNF α at the concentration of 10 ng/ml.

Our previous studies using the same culture model indicate that BDNF is not expressed by non-neuronal cells under resting conditions (Buldyrev et al., 2006). However, TNF α has been shown to stimulate BDNF expression in astrocytes (Saha et al., 2006). Thus, to rule out the possibility that the TNF α -induced increase in BDNF cell content is a result of BDNF expression by non-neuronal cells, we next examined the cellular pattern of BDNF immunoreactivity in our neuron-enriched cultures. Our data show that non-neuronal cells remain BDNF-non-immunoreactive following treatment with TNF α (Fig. 1B). This result indicates that the observed upregulation of BDNF expression in response to TNF α occurs in TG neurons.

We next addressed the possibility that the TNF α -induced upregulation of BDNF involves nerve growth factor (NGF), whose role in neuroinflammation is well established (Woolf et al., 1994, 1997), including regulation of BDNF expression (Mannion et al., 1999). We first examined immunoreactivity for NGF in cultures treated with 10 ng/ml of TNF α for 24 hrs, compared to vehicle-treated control cultures. The results of this analysis indicate that NGF is abundantly expressed by only a small subset of TG neurons under both vehicle- and TNF α -treated conditions (Vehicle, 7.34 ± 0.59 %, TNF α , 8.38 ± 1.98 %, $n=6$ cultures, $P=0.6537$; Fig. 2). Moreover, non-neuronal cells are completely lacking NGF immunoreactivity in our culture model, vehicle- or TNF α -treated (Fig. 2). We also quantified levels of NGF in the medium from TG cultures treated with 10 ng/ml of TNF α for 24 hrs, compared to vehicle-treated cultures. Consistent with the results of the immunocytochemical analysis, levels of NGF in our cultures are very low, and not affected by TNF α treatment (vehicle, 0.45 ± 0.04 pg/culture, TNF α , 0.46 ± 0.19 pg/culture, $n=12$ cultures, $P=0.9466$). Together, these data suggest that NGF does not play a role in mechanisms of TNF α -mediated upregulation of BDNF expression in TG neurons in our model.

Previous studies from our laboratory demonstrate that BDNF is significantly co-localized with CGRP (Buldyrev et al., 2006) whose role in neuroinflammation and trigeminal pain is well established. Thus, we examined the effects of TNF α on the cell content of CGRP in extracts of the same cultures that were examined for changes in BDNF cell content. Consistent with studies by Bowen and colleagues (2006), 24-hr application of TNF α (10 ng/ml) significantly increased CGRP expression (Vehicle: 125.71 ± 34.93 pg/culture vs. TNF α : 164.69 ± 49.68 pg/culture, $n=4$; 1.28 ± 0.05 increase relative to vehicle; $P<0.01$). These results indicate that BDNF and CGRP are upregulated in parallel by TNF α in TG neurons. Together with our previous data showing CGRP-dependent upregulation of BDNF release from these neurons (Buldyrev et al., 2006), the present result suggests that the two peptides interact in mechanisms of TG neuroinflammation *in vivo*.

To begin examining the molecular mechanisms of TNF α -induced upregulation of BDNF protein, we stained our TNF α -treated cultures with an antibody against the phosphorylated form of the cyclic adenosine monophosphate-responsive element binding protein (CREB), a marker of neuronal activation (Ghosh et al., 1994; Moore et al., 1996) and a transcription factor involved in regulation of BDNF expression in neurons (Tao et al., 1998). The analysis of double BDNF/phospho-CREB immunoreactivity indicates that treatment with TNF α leads to marked increases in phospho-CREB staining in the nuclei of TG neurons that are immunoreactive for BDNF (Fig. 3A). This result suggests that the mechanism of the TNF α -mediated upregulation of BDNF occurs at the transcriptional level.

Based on these data, we further explored the TNF α -mediated upregulation of BDNF protein and the underlying molecular mechanisms. Previous studies of activity-dependent regulation of the BDNF gene have demonstrated that production of specific transcripts is dependent on the type of stimulus and the signaling pathways that are activated in response to that stimulus (reviewed by Cohen and Greenberg, 2008). Thus far, the most thoroughly

characterized activity-dependent promoters of the BDNF gene are I, IV (formerly known as III), and VI (formerly known as IV; Aid et al., 2007). For example, promoters I and IV show different sensitivity of response to activation of NMDA glutamate receptors and L-type calcium channels (Tabuchi et al., 2000). Therefore, we next asked whether TNF α regulates transcription of the BDNF gene and, if so, which promoters are sensitive to this regulation.

A 4-hour application of TNF α resulted in a significant upregulation of BDNF transcripts IV and VI. Interestingly, the 1 ng/ml concentration of TNF α was sufficient to significantly increase the amount of transcript IV, but not transcript VI. This suggests a higher sensitivity of BDNF promoter IV to TNF α -mediated activation compared with promoter VI. Moreover, TNF α had no effect on transcript I (Fig. 3B). Together, these data indicate that stimulation of TG neurons with TNF α leads to an upregulation of transcription of the BDNF gene in a promoter-specific manner.

Electrical stimulation and forskolin potentiate the TNF α -mediated upregulation of BDNF expression

Inflammatory pain is associated with nociceptor sensitization and a resulting increased neuronal activity. Therefore, we used electrical field stimulation in order to mimic an increased activity of TG neurons and examine TNF α -mediated regulation of BDNF expression under those conditions.

TG neurons were electrically stimulated for 24 hrs and next treated with TNF α for either 4 hrs (BDNF mRNA) or 24 hrs (BDNF protein). Consistent with the effects of TNF α alone on BDNF transcripts, electrical stimulation (1 Hz, 0.2-ms pulses, 100 mA/culture well) followed by a 4-hr treatment with TNF α (10 ng/ml) exerted the strongest effect on the levels of BDNF transcript IV, and BDNF transcript I remained unaffected. Out of 22 experiments, TNF α -induced upregulation of BDNF transcript IV was significantly potentiated by the electrical stimulation in 8 experiments. In the remaining 14 experiments, the potentiating effect was absent. Since we were not able to identify the difference in experimental conditions between these two groups, the data were combined and, consequently, the average effect of TNF α following the electrical field stimulation was very similar to the effect of the electrical stimulation alone (Fig. 4A). This result suggests that TNF α and electrical activation utilize overlapping signaling pathways to elicit expression of BDNF mRNA.

A 24-hr application of TNF α following a 24-hr electrical stimulation resulted in a 2-fold increase in BDNF protein synthesis, compared to a 1.3-fold increase by TNF α in unstimulated sister cultures (Fig. 4B). Given that electrical stimulation itself caused only a 1.5-fold increase in BDNF synthesis, these data indicate that neuronal activation potentiates the effect of TNF α on BDNF protein synthesis. It is worth noting that electrical stimulation applied prior to the TNF α treatment was more effective than electrical stimulation delivered during the treatment (data not shown).

Neuronal depolarization leads to increased intracellular content of cAMP (Meyer-Franke et al., 1995), and forskolin, an activator of adenylate cyclase, enhances activity-dependent BDNF mRNA expression (Zafra et al., 1992). Moreover, forskolin and/or cAMP analogs have been shown to increase nociceptor sensitivity (Ferreira and Nakamura, 1979). Therefore, one potential mechanism through which electrical stimulation promotes TNF α -mediated BDNF expression in TG neurons is an increased intracellular cAMP. To begin addressing this possibility, here we examined the effects of the adenylate cyclase activator forskolin on TNF α -mediated BDNF expression.

Previous studies have indicated the need for continuous exposure to forskolin and resulting continuous activation of signal transduction pathways in mechanisms of sensory neuron sensitization (Bolyard et al., 2000). For that reason, forskolin (5 μ M) was applied to TG neuron cultures simultaneously with TNF α (10 ng/ml). A 4-hr treatment with forskolin and TNF α resulted in a strong upregulation of BDNF transcript IV that was more than three times larger than the upregulation caused by TNF α alone (TNF α + Forskolin: 1.9-fold increase vs. TNF α alone: 0.6-fold increase above vehicle; Fig. 5A). On the other hand, when forskolin alone was applied to sister cultures, it showed a strong trend toward increasing BDNF expression, but the effect was not statistically significant (Fig. 5A). Similarly to electrical stimulation, forskolin failed to increase BDNF transcript I and its effects on transcript VI were markedly weaker compared to transcript IV (data not shown). The effect of forskolin on BDNF transcripts was mimicked by 3-Isobutyl-1-methylxanthine (IBMX; 100 μ M), a non-selective inhibitor of cAMP and cGMP phosphodiesterases (data not shown). Consistent with the effects of forskolin on BDNF transcripts, a 24-hr treatment with 5 μ M forskolin and 10 ng/ml of TNF α resulted in an upregulation of BDNF protein that was more than two-thirds larger than the effect of TNF α alone (Fig. 5B). Together, these results identify a novel phenomenon of activity- and cAMP-dependent potentiation of BDNF expression induced by TNF α .

TNF α -mediated increase in BDNF mRNA expression requires tetrodotoxin (TTX)-sensitive sodium currents and p38-mitogen-activated protein kinase (MAPK) signaling

We next sought to examine the underlying cellular mechanisms of TNF α -induced BDNF expression in TG neurons. As demonstrated above, the TNF α effects on BDNF expression are sensitive to changes in neuronal activity. The level of neuronal activity is determined to a large extent by activity of voltage-dependent sodium channels, and primary sensory neurons express both TTX-sensitive and TTX-resistant sodium channels. Also, there is evidence that proinflammatory cytokines can enhance TTX-resistant currents in nociceptors, leading to their increased excitability (Binshtok et al., 2008). Therefore, we first examined the effects of TTX on TNF α -induced BDNF protein synthesis.

TTX (1.5 μ M) was added to a half of TG neuron cultures 30 min before the application of TNF α (10 ng/ml), and both agents stayed until the end of the 24-hr incubation period. The remaining sister cultures were treated with vehicle solutions and served as controls. The magnitude of the TNF α -mediated upregulation of BDNF protein was decreased in the presence of TTX. Even so, the effect of TNF α remained highly significant (relative to vehicle: TNF α , 1.40 ± 0.11 , TTX alone, 0.98 ± 0.1 , TNF α +TTX, 1.24 ± 0.11 ; $n=5$; $P<0.01$ between 'TTX alone' and 'TNF α +TTX'). These data suggest that a significant proportion of the sodium current involved in the mechanisms of TNF α -induced BDNF protein synthesis over the 24-hr period is mediated by TTX-resistant channels. Alternatively, TNF α -mediated increase in BDNF protein cell content may not directly require generation of action potentials.

Since our data presented in the previous chapter reveal potential differences in the mechanisms underlying TNF α -induced expression of BDNF protein compared to mRNA, we next examined the role of TTX-sensitive sodium channels in TNF α -dependent upregulation of BDNF mRNA, including the effects of electrical stimulation.

One half of TG neuron cultures was pre-treated with TTX (1.5 μ M) for 30 min prior to the beginning of a 24-hr electrical field stimulation, which was followed by a 4-hr treatment with TNF α (10 ng/ml) in the presence or absence of TTX. Control cultures were treated with corresponding vehicle solutions. Unlike TNF α -dependent upregulation of BDNF protein synthesis, TTX completely abolished the TNF α -induced upregulation of BDNF mRNA, both in the presence and absence of the preceding electrical stimulation (Fig. 6). Together,

these data show that *de novo* synthesis of BDNF in response to TNF α requires TTX-sensitive sodium currents.

Next, we investigated the intracellular signaling pathways through which stimulation of TG neurons with TNF α leads to *de novo* synthesis of BDNF. TNF α has previously been shown to act through various members of the MAPK family, which includes two stress-activated protein kinase sub-families, p38 and c-jun N-terminal kinase (JNK; Thalhamer et al., 2008). There are several lines of evidence that TNF α signaling in sensory neurons is mediated through p38-MAPK (Schäfers et al., 2003b; Jin and Gereau, 2006). Therefore, we considered p38-MAPK as a likely intracellular mediator of the TNF α -induced upregulation of BDNF expression. To this end, we examined the effects of the pharmacological blockade of p38-MAPK with SB203580 on TNF α -induced BDNF mRNA and protein expression in the presence and absence of the prior electrical field stimulation.

Sister cultures of TG neurons were either electrically stimulated (according to the same parameters as those used in experiments described in the previous chapter) or only incubated for 24 hrs, followed by addition of SB203580 (1 μ M) 30 min before the beginning of a 4-hr (BDNF mRNA) or 24-hr (BDNF protein) treatment with TNF α (10 ng/ml). We found that SB203580 completely abolished both BDNF mRNA and protein expression caused by TNF α (Fig. 7A,B). In addition, SB203580 significantly inhibited the increase in BDNF mRNA due to electrical field stimulation alone (Fig. 7A). This is interesting because the p38-MAPK inhibitor was applied after the cessation of the electrical stimulation. The latter result suggests that electrical field stimulation leads to a prolonged increase in the overall activity of neurons (please see the TTX data above) and a resulting activation of intracellular signaling pathways, including the p38-MAPK pathway (Fig. 8). Together, these data show that p38-MAPK intracellular signaling pathway appears to be necessary for *de novo* synthesis of BDNF in TG neurons in response to TNF α .

DISCUSSION

The present study demonstrates for the first time that TNF α stimulates *de novo* synthesis of BDNF in TG neurons, acting at the transcriptional level in a promoter-specific manner. Moreover, electrical activity and forskolin, known to stimulate synthesis of cAMP, potentiate the TNF α -induced BDNF expression, whereas TTX and a selective antagonist of p38-MAPK abolish it.

The proinflammatory cytokines, such as TNF α and IL-1 β , play an essential role in the development and maintenance of inflammatory and neuropathic pain (Schäfers and Sorkin, 2008; Basbaum et al., 2009; Costigan et al., 2009; Latremoliere and Woolf, 2009; McMahon and Malcangio, 2009). The role of TNF α in pain sensitization has been known for almost 15 years (Sorkin et al., 1997; Woolf et al., 1997). Studies have shown that TNF α is involved in thermal and mechanical hypersensitivity (Junger and Sorkin, 2000; Oprea and Kress, 2000) by modulating TTX-resistant sodium channels in primary sensory neurons (Jin and Gereau, 2006) and by promoting insertion of calcium-permeable glutamatergic AMPA receptors into membranes of spinal cord neurons (Choi et al., 2010). TNF α also induces expression of pronociceptive proteins, including cyclooxygenase-2 (Fehrenbacher et al., 2005) and the transient receptor potential vanilloid type 1 (TRPV1; Constantin et al., 2008; Khan et al., 2008). Moreover, TNF α -mediated upregulation of TRPV1 can take place at both transcriptional (Khan et al., 2008) and posttranscriptional (Constantin et al., 2008) level.

The present study identifies a novel transcriptional target of TNF α in sensory neurons, the neurotrophin BDNF (Fig. 8) whose role as a modulator of nociceptive transmission is established (Malcangio and Lessman, 2003; Merighi et al., 2008; Latremoliere and Woolf,

2009). Previous evidence for effects of cytokines on BDNF expression is scarce. In neurons, TNF α and IL-1 β increase BDNF mRNA in hypothalamic neurons *in vitro* (Rage et al., 2006; Taishi et al., 2008) and intrathecal infusion of interleukin-6 increases BDNF mRNA in dorsal root ganglia (DRG; Murphy et al., 2000). Another study showed that inhibiting TNF α activity attenuates an increase in BDNF immunoreactivity in DRG neurons induced by nucleus pulposus *in vivo* (Onda et al., 2004).

We and others have previously shown that BDNF is upregulated in primary sensory neurons by inflammation (Cho et al., 1997a,b; Kim et al., 2001; Obata et al., 2003; Qiao et al., 2008; Tarsa et al., 2010). Based on the work by Mannion and colleagues (1999), the inflammation-induced upregulation of BDNF expression has commonly thought to be mediated by nerve growth factor (NGF). In our neuron-enriched cultures, NGF was expressed by only a small fraction of TG neurons and no non-neuronal cells. Moreover, neither NGF expression nor release were significantly affected by treatment with TNF α . Furthermore, evidence from other laboratories indicates that NGF strongly upregulates BDNF transcript I (Matsuoka et al., 2007), which was not affected by TNF α in the current study. Together, these data strongly suggest that TNF α upregulates BDNF expression in an NGF-independent manner. Consistent with this notion, TNF α increased BDNF expression *via* p38-MAPK (Fig. 8), which has previously been shown to be critical for direct effects of TNF α on sensory neurons (Schäfers et al., 2003b; Jin and Gereau, 2006; Constantin et al., 2008).

Our data indicate that TNF α and electrical stimulation lead to a selective upregulation of BDNF transcripts, with transcript IV being most strongly upregulated and transcript I completely unaffected. Production of distinct BDNF transcripts may determine the function of the BDNF protein or, alternatively, provide means to regulate the stimulus-dependence of BDNF synthesis (Cohen and Greenberg, 2008). The current findings contribute to our understanding of the role of different BDNF transcripts in determining the functional specificity of the BDNF protein, including the TNF α -mediated upregulation.

Peripheral inflammation and injury are associated with the release of inflammatory mediators, including TNF α , from immune and non-neuronal cells, and TNF α activates a cascade of other cytokines and growth factors (Schäfers and Sorkin, 2008). Many studies point to the role of glia in nociceptive sensitization and in development of chronic pain (Tsuda et al., 2005; Scholz and Woolf, 2007). They indicate that a key mediator is IL-1 β . For example, release of IL-1 β from activated microglia in spinal cord inflammation is associated with an enhanced nociceptive transmission (Clark et al., 2006, 2010). In trigeminal models of inflammatory pain hypersensitivity, astroglia-derived IL-1 β induces phosphorylation of NMDA receptors (Guo et al., 2007), and glial IL-1 β released within the trigeminal ganglion modulates the excitability of A δ trigeminal nociceptors leading to a decreased mechanical hyperalgesia (Takeda et al., 2008). There is also evidence for gap junction and paracrine signaling within the trigeminal ganglion (Thalakoti et al., 2007). In our neuron-enriched TG culture model, exogenous IL-1 β does not increase BDNF expression (Balkowiec-Iskra and Balkowiec, unpublished observations). This fact rules out the possibility that the effects of TNF α are indirect, through release of IL-1 β from non-neuronal cells. Moreover, our current and previous (Buldyrev et al., 2006) studies indicate that BDNF is not expressed by non-neuronal cells, thus, the increase in BDNF expression is unlikely to be attributed to the non-neuronal source.

This study reveals the previously unknown role of cAMP in regulating cytokine-mediated BDNF expression. These data are consistent with the previously shown effects of activating adenylate cyclase on neuronal expression of neurotrophin mRNAs induced by conventional transmitters and neuropeptides (Zafra et al., 1992). It has previously been demonstrated that central, but not peripheral, neurons require activity or increased intracellular cAMP for their

responsiveness to trophic factors (Meyer-Franke et al., 1995, 1998; Shen et al., 1999; Goldberg et al., 2002), in part through an increased surface expression of the BDNF receptor TrkB (Meyer-Franke et al., 1998). Our current data indicate that neuronal activity and cAMP enhance responsiveness of sensory neurons to cytokines that, in turn, leads to an augmented BDNF expression (Fig. 8). This mechanism could play an important role in axonal regeneration after injury, which is associated with inflammation and release of proinflammatory cytokines, such as TNF α .

TG neurons express both types of TNF α receptors and the role of TNF α as a neuromodulator in the trigeminal system has been proposed several years ago (Cunningham et al., 1997). Studies in DRG show that expression of TNF α and its receptors can be strongly upregulated by inflammation and injury in both neurons and non-neuronal cells (Schäfers et al., 2003a; Li et al., 2004; Schäfers et al., 2008), and the neuronal expression of TNF α increases after nerve injury (Shubayev and Myers, 2001). These data suggest that neuron- and non-neuronal cell-derived TNF α could act in an auto- or paracrine fashion to upregulate BDNF expression in TG neurons. For example, our previous study showed that inflammation within just two teeth leads to a widespread upregulation of BDNF expression in TG neurons (Tarsa et al., 2010), the phenomenon which could potentially be explained by upregulation of TNF α receptors in not only inflamed, but also non-inflamed neurons and/or paracrine action of TNF α on these neurons. In support, spinal nerve ligation leads to sensitization of both injured and adjacent uninjured DRG neurons to TNF α (Schäfers et al., 2003a).

Our previous studies point to BDNF as a likely modulator of nociceptive transmission at first-order synapses in trigeminal pathways (Buldyrev et al., 2006; Tarsa et al., 2010). TG neurons constitute the first link of nociceptive information processing in such chronic pain conditions as migraine, trigeminal neuralgia and temporomandibular joint disorders. There is evidence that TNF α is significantly increased in plasma and cerebrospinal fluid of migraineurs and patients with daily persistent headaches (Perini et al., 2005; Rozen and Swidan, 2007). Our current data offer a scenario where TNF α increases BDNF expression in TG neurons, leading to trigeminal hyperalgesia. In support, peripheral inflammation enhances BDNF signaling in central pain modulatory circuitry (Guo et al., 2006; Ren and Dubner, 2007). However, the physiological significance of the TNF α -mediated upregulation of BDNF in TG neurons remains to be determined.

There is increasing evidence that cytokines are involved in normal neuronal development and plasticity. The current data show that TNF α activates the transcription factor cAMP-response element-binding protein (CREB), which is essential for the activity-dependent transcription of the BDNF (Shieh et al., 1998; Tao et al., 1998; West et al., 2001) and other synaptic plasticity genes (Ji et al., 2003). Other studies have identified immune cells and microglia as critical for the maintenance of adult neurogenesis and learning abilities. Moreover, the immune T cells have been shown to be required for the expression of BDNF in the hippocampus (Ziv et al., 2006).

Together, our findings provide a new evidence for a link between the nervous and immune systems through TNF α -evoked *de novo* synthesis of BDNF in neurons. These data have implications for understanding mechanisms of neuroinflammatory disorders of the central and peripheral nervous system, as well as normal neuronal development and plasticity.

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LIST OF ABBREVIATIONS

BDNF	brain-derived neurotrophic factor
CGRP	calcitonin gene-related peptide
CREB	cAMP response element-binding protein
pCREB	phosphorylated cAMP response element-binding protein
DAPI	4',6-diamidino-2-phenylindole
ELISA	enzyme-linked immunosorbent assay
IBMX	3-Isobutyl-1-methylxanthine
IL-1β	interleukin-1 β
MAPK	mitogen-activated protein kinase
NGF	nerve growth factor
RT-PCR	reverse-transcription polymerase chain reaction
TG	trigeminal ganglion
NF	neurofilament
TNFα	tumor necrosis factor- α
TTX	tetrodotoxin

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Figure 1. Tumor necrosis factor- α (TNF α) increases BDNF protein expression in cultured newborn rat trigeminal ganglion (TG) neurons

A, Mean BDNF cell content (pg/culture) of TG cultures treated for 24 hrs with vehicle (Veh) or TNF α (1, 10 and 50 ng/ml); n=5 experiments; at least 2 cultures per experiment per group; * p<0.05; ** p<0.01. **B**, Representative neuron-enriched dissociate culture of newborn rat TG treated for 24 hrs with TNF α (10 ng/ml), followed by double-immunostaining for BDNF (α BDNF, top panel) and neurofilament (red) with DAPI (blue) counterstain (α NF/DAPI, bottom panel); scale bar, 50 μ m.

Figure 2. Immunoreactivity for nerve growth factor (NGF), which is expressed by a small fraction of TG neurons and none of non-neuronal cells, is not affected by TNF α
 Representative neuron-enriched dissociate cultures of newborn rat TG treated for 24 hrs with vehicle (Veh) or TNF α (10 ng/ml). The cultures were double-immunostained for nerve growth factor (α NGF, green, top panel) and neurofilament (red) with DAPI (blue) counterstain (Overlay, bottom panel). Arrows indicate cells that express double NGF/neurofilament immunoreactivity; scale bar 50 μ m.

Figure 3. Tumor necrosis factor- α (TNF α) stimulates BDNF expression at the transcriptional level

A, Representative neuron-enriched dissociate cultures of newborn rat TG treated for 24 hrs with vehicle (Veh) or TNF α (10 ng/ml), followed by double-immunostaining for the phosphorylated form of the cyclic adenosine monophosphate-responsive element binding protein (α pCREB, green, top panel) and brain-derived neurotrophic factor (α BDNF, red, bottom panel). Arrows indicate cells that show strong pCREB immunoreactivity in the nucleus (top panel) and BDNF immunoreactivity in the cytoplasm (bottom panel); scale bar 50 μ m. **B**, Levels of three activity-dependent BDNF transcripts (I, IV, VI) in response to 4-hr treatment of sister cultures with increasing concentrations of TNF α (1, 10 or 50 ng/ml), normalized in relation to levels of the endogenous reference gene transcript hypoxanthine-guanidine phosphoribotransferase (HPRT), and presented relative to vehicle (Veh); n=4 experiments. *p<0.05, **p<0.01.

Figure 4. Electrical stimulation of TG neurons results in potentiation of TNF α -induced BDNF protein expression

A, Levels of three BDNF transcripts (I, IV and VI; relative to unstimulated vehicle; Ctrl-Veh, white solid bar) in response to 4-hr treatment of cultures with 10 ng/ml TNF α (Ctrl-TNF α , grey solid bar), 24-hr electrical field stimulation at 1 Hz followed by 4-hr treatment with vehicle (Stim-Veh, white hatched bar), or 24-hr electrical stimulation followed by 4-hr treatment with 10 ng/ml TNF α (Stim-TNF α , grey hatched bar). Individual transcript levels were calculated using the reference gene HPRT; n=22 experiments. **B**, BDNF cell content (relative to unstimulated vehicle whose value was made equal to one; Ctrl-Veh, white solid bar) in response to 24-hr treatment with 10 ng/ml TNF α alone (Ctrl-TNF α , grey solid bar), 24-hr electrical field stimulation at 1 Hz followed by 24-hr treatment with vehicle (Stim-Veh, white hatched bar), or 24-hr stimulation followed by 24-hr treatment with 10 ng/ml TNF α (grey hatched bar); n=5 experiments. *p<0.05, **p<0.01, ***p<0.001.

Figure 5. Forskolin, an activator of adenylate cyclase, potentiates the TNF α -mediated increase in BDNF transcription

A, Levels of BDNF transcript IV (relative to vehicle whose value was made equal to one, white solid bar) in response to 4-hr treatment with 10 ng/ml TNF α alone (grey solid bar), 5 μ M forskolin alone (white striped bar) or 10 ng/ml TNF α combined with 5 μ M forskolin (grey striped bar). Transcript levels were calculated using levels of the reference gene HPRT; n=7 experiments. **B**, BDNF cell content (relative to vehicle whose value was made equal to one; white solid bar) in response to 24-hr treatment with 10 ng/ml TNF α alone (grey solid bar), 5 μ M forskolin alone (white striped bar) or 10 ng/ml TNF α combined with 5 μ M forskolin (grey striped bar); n=3 experiments. *p<0.05, **p<0.01, ***p<0.001.

Figure 6. Tetrodotoxin (TTX), an inhibitor of voltage-activated sodium channels, abolishes the TNF α - and electrical stimulation- mediated upregulation of BDNF transcription in TG neurons Levels of BDNF transcript IV (relative to unstimulated vehicle whose value was made equal to one, Veh, white solid bars) in response to 4-hr treatment of cultures with 10 ng/ml TNF α (TNF α , grey solid bars), 24-hr electrical field stimulation at 1 Hz followed by 4-hr treatment with vehicle (Stim-Veh, white hatched bars), or 24-hr electrical stimulation followed by 4-hr treatment with 10 ng/ml TNF α (Stim-TNF α , grey hatched bars), in the absence (Ctrl) and presence of 1.5 μ M TTX (TTX). Individual transcript levels were calculated using the reference gene HPRT; n=4 experiments. *p<0.05, **p<0.01, ***p<0.001.

Figure 7. Inhibition of the p38 MAPK pathway abolishes the TNF α -induced upregulation of BDNF transcription in TG neurons, in the absence or presence of electrical stimulation

A, Levels of BDNF transcript IV (relative to unstimulated vehicle whose value was made equal to one, Veh, white solid bars) in response to a 4-hr treatment of cultures with 10 ng/ml TNF α (TNF α , grey solid bars), 24-hr electrical field stimulation at 1 Hz followed by 4-hr treatment with vehicle (Stim-Veh, white hatched bars), or 24-hr electrical stimulation followed by 4-hr treatment with 10 ng/ml TNF α (Stim-TNF α , grey hatched bars), in the absence (Ctrl) or presence of an inhibitor of p38 MAPK (SB 203580, 1 μ M). Individual transcript levels were calculated using the reference gene HPRT; n= 6 experiments. **B**, BDNF cell content (relative to vehicle whose value was made equal to one; white solid bars) in response to a 24-hr treatment with 10 ng/ml TNF α (grey solid bar) in the absence (Ctrl) or presence of an inhibitor of p38 MAPK (SB 203580, 1 μ M); n=4 experiments. *p<0.05, **p<0.01.

Figure 8. Proposed model of TNF α -mediated regulation of BDNF expression in neurons

TNF α , released from macrophages or microglial cells, stimulates the p38 mitogen-activated protein kinase (p38 MAPK) pathway to increase phosphorylation (P) of cAMP response element (CRE)-binding (CREB), a transcription factor known to regulate the BDNF gene. In turn, CREB phosphorylation leads to activation of exon IV of the BDNF gene, and subsequent BDNF mRNA and protein synthesis. Neuronal depolarization, dependent on tetrodotoxin-sensitive (TTX-S) sodium channels, increases intracellular cAMP, which acts through several putative mechanisms to potentiate the TNF α regulation of BDNF transcription. For example, cAMP activates protein kinase A (PKA), a well-established mechanism of CREB phosphorylation. In addition, cAMP is a likely mediator of depolarization-induced upregulation of p38 MAPK activity.