Published in final edited form as:

Arch Oral Biol. 2017 March ; 75: 100–106. doi:10.1016/j.archoralbio.2016.10.034.

Tumor Necrosis Factor-Alpha Stimulates Cytokine Expression and Transient Sensitization of Trigeminal Nociceptive Neurons

Zachary L. Durham, MS^a, Jordan L. Hawkins, MS^a, and Paul L. Durham, PhD^a

^aMissouri State University, JVIC-CBLS, 524 North Boonville Avenue, Springfield, MO 65806, Phone: 417-836-4869

Abstract

Objective—Elevated levels of tumor necrosis factor-alpha (TNF- α) in the capsule of the temporomandibular joint (TMJ) are implicated in the underlying pathology of temporomandibular disorders (TMD). TMD are a group of conditions that result in pain in the TMJ and/or muscles of mastication, and are associated with significant social and economic burdens. The goal of this study was to investigate the effect of elevated TNF- α levels in the TMJ capsule on nocifensive behavioral response to mechanical stimulation of trigeminal neurons and regulation of cytokines within the trigeminal ganglion.

Design—Male Sprague-Dawley rats were injected bilaterally in the TMJ capsule with TNF- α and changes in nocifensive head withdrawal responses to mechanical stimulation of cutaneous tissue directly over the capsule was determined using von Frey filaments. Cytokine levels in trigeminal ganglia were determined by protein array analysis at several time points post injection and correlated to nocifensive behavior.

Results—TNF- α caused a significant increase in the average number of nocifensive responses when compared to naive and vehicle treated animals 2 hours post injection, but levels returned to control levels at 24 hours. Based on array analysis, the levels of eight cytokines were significantly elevated above vehicle control levels at 2 hours following TNF- α injection, but all eight had returned to the vehicle control levels after 24 hours.

Conclusions—Our findings provide evidence that elevated levels of TNF- α in the joint capsule, which is reported to occur in TMD, promotes nociception in trigeminal ganglia neurons via a mechanism that temporally correlates with differential regulation of several cytokines.

Please address correspondence to: Paul L. Durham, Phone: 417-836-4869, Fax: 417-836-7602.

Conflict of interest

None.

Ethical approval

Approved by Missouri State University's Institutional Animal Care and Use Committee and conducted in compliance with all established guidelines in the Animal Welfare Act of 2007, National Institutes of Health, and ARRIVE Guidelines.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

Temporomandibular disorder (TMD); tumor necrosis factor-alpha (TNF- α); trigeminal; nocifensive; sensitization

1. Introduction

More than 11 million American adults suffer from symptoms attributed to temporomandibular disorders (TMD) including jaw pain, joint pain, muscle pain, restricted movement of the jaw, headache, tinnitus, dizziness, hearing loss, and swelling of the jaw (Mujakperuo, Watson, Morrison, & Macfarlane, 2010). TMD predominantly affects women of childbearing age and is the second most common cause of orofacial pain only after dental pain (Manfredini et al., 2011; Poveda Roda, Bagan, Diaz Fernandez, Hernandez Bazan, & Jimenez Soriano, 2007). In addition, individuals suffering from TMD often exhibit increased sensitivity to other experimentally induced pains (Greenspan et al., 2011a). Given the significant morbidity of TMD, it is understandable that these disorders have significant social and economic ramifications (Slade et al., 2011). Increased expression of tumor necrosis factor-alpha (TNF- α), which is a pro-inflammatory cytokine, promotes the initiation and progression of multiple inflammatory diseases, including TMD (Alstergren, 2000; Nishimura, Segami, Kaneyama, Suzuki, & Miyamaru, 2002; Taylor, Williams, & Feldmann, 2004). In support of this notion, elevated TNF- α levels in the jaw or temporomandibular joint (TMJ) positively correlate with acute and chronic joint inflammation, connective tissue destruction, and pain in TMD (Emshoff, Puffer, Rudisch, & Gassner, 2000; Fredriksson, Alstergren, & Kopp, 2006; Fu, Ma, Zhang, & Chen, 1995; Kacena et al., 2001; Nordahl, Alstergren, & Kopp, 2000). Furthermore, inhibition of TNF- α is a therapeutic strategy for treating symptoms associated with TMD pathology (Kristensen et al., 2009; Ohtani et al., 2012; Stoustrup et al., 2009). Although the specific pathophysiology associated with the TMD has yet to be fully elucidated, cytokines including TNF- α have been linked with synovial inflammation and connective tissue degradation in joints (Lobbezoo et al., 2004).

In response to injury or infection, TNF- α is released within the TMJ by both immune and non-immune cells such as macrophages and synoviocytes, and neurons associated with the trigeminal ganglion (Leung & Cahill, 2010). The trigeminal nerve, which provides sensory innervation of the TMJ and associated masticatory structures, is responsible for transmitting nociceptive signals from the peripheral tissues to the upper spinal cord (Imbe et al., 2001; Shankland, 2000). While the cell bodies of trigeminal sensory neurons are located in the trigeminal ganglion, these neurons extend fibers to peripheral tissues in the head and face and extend fibers that project centrally and synapse with second order neurons in the spinal trigeminal nucleus (Cairns, Sessle, & Hu, 2001; Nishimori et al., 1986; Shigenaga et al., 1988). Within the trigeminal ganglion, the neuronal cell body is surrounded by satellite glial cells that modulate the excitability state of trigeminal ganglion neurons (Durham & Garrett, 2010; Thalakoti et al., 2007). Following activation of trigeminal neurons, cytokine release within the ganglion promotes increased neuron-glia communication via paracrine signaling and gap junctions that establish an inflammatory loop associated with peripheral

sensitization (De Corato et al., 2011; Takeda et al., 2007). The development of sensitized nociceptive neurons, which exhibit lower activation thresholds, is a key component of TMD pathology (Ren & Dubner, 2008; Sessle, 2011). TNF- α is known to promote peripheral sensitization of nociceptive neurons by modulating the expression and activity of ion channels and receptors and causing excitation of primary sensory neurons (Cheng & Ji, 2008).

TNF- α is thought to play a central role in the development of TMD (Fu et al. 1995) by promoting inflammation within the joint, and causing activation of nociceptive trigeminal ganglion neurons. While it is known that higher levels of TNF- α in the TMJ capsule are associated with increased pain, the inflammatory cytokines upregulated in response to TNF- α in the TMJ capsule has not been investigated. The goal of this study was to investigate the effect of elevated levels of TNF- α in the TMJ capsule, as reported in association with TMD pathology, on nocifensive behavioral response to mechanical stimulation of trigeminal neurons and the regulation of other cytokines within the trigeminal ganglion.

2. Materials and methods

2.1 Animals

All protocols were approved by Missouri State University's Institutional Animal Care and Use Committee and conducted in compliance with all established guidelines in the Animal Welfare Act of 2007, National Institutes of Health, and ARRIVE Guidelines. Concerted efforts were made to minimize suffering, as well as the number of animals used in this study ($n = 30$). Adult, male Sprague-Dawley rats (350 – 500 g; Charles River Laboratories Inc., Wilmington, MA) were housed in clean, plastic standard rat cages (VWR, West Chester, PA) in an animal holding room on a 12-hour light/dark cycle starting at 7 A.M. with ambient temperature maintained from 22–24°C and access to food and water *ad libitum*. Animals were acclimated to the environment for a minimum of one week upon arrival prior to use.

2.2 Materials

A stock solution of 100 ng/mL TNF- α was made using 20 μ g of Recombinant Rat TNF- α (Thermo Scientific, Rockford, IL) and 1X Phosphate Buffered Saline (PBS) (Sigma-Aldrich, St. Louis, MO). On the day of the experiment, an aliquot of 100 ng/mL TNF- α was thawed and a sterile solution of PBS was made in deionized water as per manufacturer's instructions to serve as the vehicle control.

2.3. Effect of TNF- α on nocifensive head withdrawal response to mechanical stimulation

The method used in this study was based on our published studies using the Durham Animal Holder (Ugo Basile, Varese Lakes, Italy) to facilitate measurement of nocifensive response in the head and face of rats to mechanical stimulation (Cady, Denson, Sullivan, & Durham, 2014; Garrett, Hawkins, Overmyer, Hayden, & Durham, 2012; Hawkins, Denson, Miley, & Durham, 2015). Animals freely entered the holder and were allowed to remain in the device for 5 minutes on 3 consecutive days. The animals were secured in the optimal position for testing mechanical sensitivity in the orofacial region and minimizing limb movement using a plastic restraining block that was inserted behind the animal. During this acclimation period,

if a rat appeared to be unwilling to go into the device or was continuously moving and shifting within the device, the animal was removed from the study. To minimize false responses, animals were conditioned to a mechanical stimulus by gently rubbing the hair follicles and epidermis located over each TMJ with a pipette tip. Initially, baseline mechanical nocifensive thresholds were determined in response to a series of calibrated von Frey filaments (North Coast Medical, Inc., Gilroy, CA; 15, 26, 60, 100, 180, 300 grams) applied in increasing force to the cutaneous area over the right and left TMJ.

The researcher responsible for directly testing the response to each filament was blinded to the experimental conditions. A positive response, which was defined by head withdrawal prior to the bending of the filament, was recorded by a second researcher. Each filament was applied 5 times on each side, and the data are reported as the average number of head withdrawal responses obtained from 10 applications. The 100 g force was chosen for subsequent studies since the average number of basal positive head withdrawal responses to this force for each experimental group of animals was consistently less than 1 out of 5 for both right and left TMJ, while the 180 g force elicited a response over 3 out of 5 for both the right and left TMJ. Following measurement of basal mechanical withdrawal responses, the animals were anesthetized by inhalation of 5% isoflurane. The animals were injected in both left and right TMJ with 50 μ L of either 100 ng/mL TNF- α (n=6) or PBS (n=6), which served as a vehicle control. The concentration of 100 ng/ml TNF- α was chosen based on previously published studies in which that same concentration of TNF- α was reported to stimulate neuropeptide release from cultured trigeminal ganglion cells (Bowen, Schmidt, Firm, Russo, & Durham, 2006). The concentration of 100 ng/ml is also within the range reported in the synovial fluid of TMD patients (Fu et al., 1995). Other male Sprague-Dawley rats were left untreated to serve as naïve controls (n=6). The animals were injected using a 26½ G needle (Becton Dickinson, Franklin Lakes, NJ) and a 50 L Hamilton syringe (Hamilton Company, Reno, NV). At 2 and 24 hours following injection, the number of nocifensive head withdrawals to mechanical stimulation was determined in the manner as performed to record the animal's basal readings.

2.4. TNF- α regulation of other cytokines in trigeminal ganglion

For these studies, animals were left untreated (naïve control, n=3) or injected with either PBS (n=3) or 100 ng/ml TNF- α (2hr: n=3; 24hr: n=3) and tissues collected from animals sacrificed by CO₂ asphyxiation and decapitation. Both trigeminal ganglia were removed and frozen in liquid nitrogen. The tissues were homogenized via sonication in 100 μ L of cell lysis buffer (RayBiotech, Norcross, GA) diluted 1:1 in PBS, and centrifuged at 3,200 rpm for 20 minutes at 4°C. The supernatant was transferred into a 1.5 mL tube and stored at -20°C for protein array analysis.

Experimental lysates were analyzed using rat cytokine arrays (Proteome Profiler, R&D Systems, Minneapolis, MN) following manufacturer's instructions. The total amount of protein in each sample was determined by the Bradford method, and for each experimental condition 200 μ g of protein was used for analysis. Detection of immunoreactivity was performed using a chemiluminescent peroxidase substrate (Pierce ECL Plus Western Blotting Substrate, Thermo Scientific, Waltham, MA). Silver halide film (Kodak BioMax

Film, Sigma-Aldrich, St. Louis, MO) was exposed to the chemiluminescent blots for several different exposure times to provide a range of dot densities for semi-quantitative analysis.

Results were analyzed using the protein array analyzer macro for Image J software (Schneider, Rasband, & Eliceiri, 2012). A 2D rolling ball background subtraction with a radius of 40 was used to normalize background levels. The pixel density of three pairs of dots was determined from blots with a short (10 seconds), medium (30 seconds), or long (2 minutes) exposure time based on their relative intensity. Three different exposure times were used so that the dots would be dark enough to measure density without being overexposed. Circles with a diameter of 7 pixels were placed over each dot on the protein array to determine pixel density. For analysis, the average pixel density of the background was subtracted from the pixel density for each dot.

2.5 Statistical analysis

For the nocifensive behavioral studies, the data are reported as the combined average number of head withdrawal responses $\pm$ SEM to 100 g of force at each condition and time point. Subsequent analysis was then performed on data with $n = 6$ for each experimental condition by using a non-parametric Kruskal Wallis followed by the Mann-Whitney U posthoc test at each individual time point because collected data were shown to have unequal variance as determined by Levene's test and failed a Shapiro-Wilk test for normality. All analysis was conducted using SPSS 21.0.0 software (IBM, Armonk, NY). Differences were considered significant at $P < 0.05$. For the cytokine studies, data are presented as average fold change $\pm$ SEM ($n = 3$ animals per condition) when compared to values obtained from control or vehicle injected animals, whose means were set equal to 1. Data points were excluded from analysis that showed high variance between the two duplicate points, undetectable points, and points with overlapping intensities. Statistical analysis was performed using a non-parametric Mann-Whitney U test using SPSS 21.0.0 software due to the fact that collected data were shown to have unequal variance as determined by Levene's test. Subsequent analysis was then performed on data with $n = 3$ or greater for each experimental condition. Statistical significance was set at $P < 0.05$. All analysis was performed using SPSS.

3. Results

3.1. TNF- α promotes a transient increase in nocifensive response to mechanical stimulation

The average number of head withdrawal responses to mechanical stimulation of the cutaneous tissue above both TMJ for each experimental condition is reported in Figure 1. Animals injected bilaterally with TNF- α exhibited a significant ($P < 0.05$) increase in the number of head withdrawal responses 2 hours post joint injection when compared to average values for the naïve control or vehicle conditions. However, the number of withdrawal responses mediated by TNF- α was similar to naïve and vehicle control levels after 24 hours. Injection of PBS, which served as the vehicle control, did not cause a significant increase over naïve levels at either time point.

3.2. TNF- α differentially regulates cytokine expression in trigeminal ganglia

The relative expression of inflammatory cytokines in the trigeminal ganglion was analyzed using a commercially available cytokine array under basal naïve conditions and in animals injected bilaterally with PBS or TNF- α . Of the 29 cytokines measured, seven were excluded from further analysis due to their average values being more than two standard deviations from the mean (data not shown). The 7 cytokines that were not evaluated due to high variability were CINC-3, IL-1ra, IL-2, IL-13, IL-17, RANTES, and TNF- α .

When compared to naïve control, the fold change in the levels of 8 cytokines following injection with the vehicle PBS were significantly ($P < 0.05$) elevated between 1.5 and 3.25 fold, while 2 cytokines were elevated between 3.25 and 5 fold (Fig. 2). None of the cytokines were increased > 5 fold by vehicle control when compared to naïve control. For the vehicle control, CINC-2 α/β , fractalkine, GM-CSF, INF- γ , IL-1 α , IL-6, IL-10, IP-10, L-selectin, and MIP-1 α were all significantly elevated when compared to naïve control levels in the trigeminal ganglion. In contrast, the levels of CNTF and thymus chemokines were significantly decreased.

In response to TNF- α 2 hours after injection, there were 4 cytokines whose mean levels were significantly increased between 1.5 and 3.25 fold, 3 cytokines between 3.25 and 5 fold, and 11 cytokines > 5 fold compared to naïve control (Fig. 3). For TNF- α at 24 hours post injection, there were 10 cytokines with significantly elevated levels between 1.5 and 3.25 fold, one between 3.25 and 5 fold, and none > 5 fold when compared to the naïve control. TNF- α at 2 hours stimulated significant increases in the expression of CINC-1, CINC-2 α/β , fractalkine, GM-CSF, INF- γ , IL-1 α , IL-1 β , IL-3, IL-4, IL-6, IL-10, IP-10, LIX, L-selectin, MIG, MIP-3 α , TIMP-1, and VEGF in trigeminal ganglia when compared with naïve control levels. At 24 hours post injection, TNF- α significantly elevated the levels of fractalkine, GM-CSF, INF- γ , IL-1 α , IL-3, IL-4, IL-6, IL-10, IP-10, L-selectin, MIG and VEGF, when compared to naïve control levels in the trigeminal ganglia. In contrast, the level of TIMP-1 was significantly decreased in response to TNF- α . A summary of the relative fold change $\pm$ SEM for all regulated cytokines when compared to naïve control values is shown in Table 1.

In addition, the cytokine levels in the trigeminal ganglia in response to TNF- α at 2 and 24 hours were compared to the vehicle control levels. As seen in Figure 4, differences in fold change are reported for the number of cytokines significantly ($P < 0.05$) elevated in response to TNF- α versus vehicle treated animals. For TNF- α 2 hours after injections, there were 3 cytokines whose levels were increased greater than 1.5 and 2 fold, 3 between 2 and 2.5 fold, and 2 > 2.5 fold that were significantly elevated compared to vehicle control in the trigeminal ganglion. At 24 hours post injection, there was 1 cytokine greater than 1.5 and 2 fold, 1 between 2 and 2.5 fold, and none greater than 2.5 fold that were significantly elevated compared to vehicle control in the trigeminal ganglion. The cytokines CNTF, fractalkine, IL-1 α , IL-1 β , IL-6, IP-10, thymus chemokines, and VEGF were significantly ($P < 0.05$) elevated over vehicle control after 2 hours, while only CNTF and thymus chemokines were significantly elevated compared to vehicle control in the trigeminal ganglion 24 hours after TNF- α injection. In addition, the level of several cytokines including CINC-1, INF- γ , IL-6, MIP-3 α , and TIMP-1 were significantly decreased at the 24 hour time point. A summary of

the relative fold change $\pm$ SEM for all regulated cytokines when compared to vehicle control values is shown in Table 2.

4. Discussion

We found that bilateral injection of TNF- α , which mimics the elevated level reported in male and female patients with TMJ pathology, caused a transient increase in nociception and differential cytokine regulation in the trigeminal ganglion. The inclusion of only male animals may be a potential limitation of our study. However, to our knowledge this is the first study to investigate behavioral and cellular changes in response to elevated TNF- α levels in the TMJ capsule as has been reported in humans with TMD. Our model was based on the evidence that elevated TNF- α levels within the synovial fluid obtained from the joint capsule directly correlate with TMJ inflammation, pain, and tissue destruction in these individuals (Fu et al., 1995; Shafer, Assael, White, & Rossomando, 1994; Spears, Dees, Sapozhnikov, Bellinger, & Hutchins, 2005). Insufficient systemic control of TNF- α can also cause pain and tissue destruction in the TMJ of patients suffering from rheumatoid arthritis (Alstergren & Kopp, 2006). Elevated levels of TNF- α have been reported in the cell layer of the synovial lining and blood vessels of the TMJ, and hence, thought to have a role in internal derangement of the TMJ (Suzuki, Segami, Nishimura, & Nojima, 2002). In addition, TNF- α levels in the synovial fluid positively correlate with TMJ pain during movement and tenderness upon posterior palpation of the joint (Nordahl et al., 2000). Also, a direct correlation was shown between TNF- α levels and the preoperative TMJ pain in joint lavage of the patients with internal derangement of the TMJ (Shafer et al., 1994). Thus, our finding that higher levels of TNF- α can promote sensitization and activation of trigeminal nociceptive neurons is in agreement with data from human studies.

To better understand the temporal response of TNF- α in TMD pathology, rats were injected bilaterally in the TMJ capsule with TNF- α and mechanical stimulation of the TMJ was used to evaluate the rat's nocifensive response. Rats injected with TNF- α 2 hours prior to mechanical stimulation demonstrated a significant increase in the number of head withdrawal responses to cutaneous stimulation over the TMJ when compared to naïve and vehicle treated animals. We found that the nocifensive effect of TNF- α was similar to naïve and vehicle control animal responses 24 hours post injection. These results provide evidence that TNF- α had an acute transient sensitizing effect on the trigeminal neurons that provide sensory innervation of the TMJ. Our findings are in agreement with the results of a prior study that reported that elevated TNF levels correlated with increased nociception (Song, Li, Tang, Huang, & Yuan, 2014). In contrast to the stimulatory effect of TNF- α , injection of the same volume of vehicle solution in the capsule did not cause a significant increase in nocifensive behaviors. Since cytokines are known to promote peripheral sensitization by increasing the activity of pronociceptive receptors and ion channels in sensory neurons (Leung & Cahill, 2010), the effects of TNF- α injection on cytokine levels in the trigeminal ganglion was investigated.

In response to elevated TNF- α concentration in the TMJ capsule, the levels of some but not all cytokines were upregulated in a manner that temporally correlated with an increased nocifensive response. The level of 18 cytokines in the trigeminal ganglion were significantly

elevated over naïve control levels 2 hours post TNF- α injection, while the level of 11 cytokines remained significantly elevated 24 hours post injection. In addition to the naïve control, we investigated changes in cytokine levels that might occur due to increased pressure within the capsule caused by injection of 50 μ l of fluid since an increase in fluid volume in the capsule, referred to as edema, has been reported to occur in TMD (Villa et al., 2010). Following injection with the vehicle control, we found that the levels of 10 cytokines were elevated over naïve control values. However, elevated levels of these cytokines did not elicit an increase in the number of nocifensive head withdrawals and thus are not likely involved in mediating a nocifensive behavioral response. We can only speculate that the cytokines upregulated by the increased volume in the capsule may increase the sensitivity of the trigeminal neurons to other types of stimulation. The TMJ contains free nerve endings and sensory nerve end organs around the disk of parenchyma that function as proprioceptors that respond to changes in pressure/tension within the joint capsule. Activation of these proprioceptors, as would occur during tissue swelling/edema, is implicated in the increased sensitivity that patients experience when suffering from TMD (Asaki, Sekikawa, & Kim, 2006). We found that only 8 cytokines were significantly elevated over vehicle control levels 2 hours after injection with TNF- α , the time point that correlates with increased nociception. In contrast, the levels of only 2 cytokines, CNTF and thymus chemokines, remained elevated over vehicle control 24 hours post injection. Based on our findings, we propose that increased levels of CNTF, fractalkine, IL-1 α , IL-1 β , IL-6, IP-10, thymus chemokines and VEGF in the trigeminal ganglion at 2 hours are likely to be involved in promoting nociception in response to elevated levels of TNF- α in the TMJ capsule. Furthermore, we speculate that the sustained increase in CNTF and thymus chemokines 24 hours post TNF- α injection are likely to contribute to sensitization of trigeminal nociceptive neurons.

The mechanism by which TNF- α increases nociception and cytokine expression within the trigeminal ganglion is likely to involve activation of TNF- α receptors, which are known to be expressed on trigeminal neurons, and the stimulated release of CGRP from neuronal cell bodies (Bowen et al., 2006). CGRP has been shown to increase the release from satellite glial cells of cytokines known to promote and sustain an inflammatory loop characterized by enhanced sensitization and nociception (Capuano et al., 2009; De Corato et al., 2011). For several of the cytokines including IL-1 β and IL-6 there is evidence to support their role in the activation of trigeminal neurons or satellite glial cells (Neeb et al., 2011; Yan, Melemedjian, Price, & Dussor, 2012; Zhang, Burstein, & Levy, 2012). In addition, fractalkine is reported to contribute to the genesis of inflammatory nociception during peripheral inflammation that is likely to be dependent on the activation of the satellite glial cells, which results in the production of other cytokines that are involved in maintenance of an inflammatory state (Souza et al., 2013). However, the potential contribution of CNTF, IL-6, IP-10, thymus chemokines and VEGF has not yet been investigated but likely involves stimulation of neurons and/or satellite glial cells. In support of this notion, CNTF, is a member of the interleukin-6 family and has been shown to be a neuroprotective cytokine that promotes survival of neurons and glia in response to neural inflammation (Linker et al., 2002). IP-10 is a chemokine that is a chemoattractant for macrophages (Kasama et al., 2005). We have recently reported that the number of resident macrophages within the trigeminal ganglion is increased in response to inflammation of the TMJ (Cady et al., 2014).

It is possible that IP-10 may be playing a role in recruiting additional macrophages into the ganglion in response to joint inflammation and subsequent activation of trigeminal neurons. We can only speculate that elevated levels of VEGF and thymus cytokines likely are involved in promoting inflammation within the ganglion by increasing neuron-glia signaling via activation of other cytokines (Croll et al., 2004).

5. Conclusions

We found that elevated levels of TNF- α , as reported in the synovial fluid obtained from the TMJ capsule of TMD patients, caused an increase in the nocifensive response to mechanical stimulation of the cutaneous tissue above the TMJ that temporally correlated with increased expression of several cytokines. Based on our findings, we propose that the pronociceptive effect of TNF- α in TMD pathology is mediated, at least in part, via differential expression of proinflammatory cytokines within the ganglion to promote sensitization of trigeminal neurons that provide sensory innervation of the TMJ capsule.

Acknowledgments

We would like to thank Allison Overmyer and Jennifer Cashler for their assistance with animal husbandry.

Funding

Supported by grant from National Institutes of Health (DE017805).

Abbreviations

CGRP	Calcitonin gene-related peptide
PBS	Phosphate buffered saline
TMJ	Temporomandibular joint
TMD	Temporomandibular disorders

References

- Alstergren P. Cytokines in temporomandibular joint arthritis. *Oral Dis.* 2000; 6(6):331–334. [PubMed: 11355265]
- Alstergren P, Kopp S. Insufficient endogenous control of tumor necrosis factor-alpha contributes to temporomandibular joint pain and tissue destruction in rheumatoid arthritis. *J Rheumatol.* 2006; 33(9):1734–1739. [PubMed: 16960936]
- Asaki S, Sekikawa M, Kim YT. Sensory innervation of temporomandibular joint disk. *J Orthop Surg (Hong Kong).* 2006; 14(1):3–8. [PubMed: 16598078]
- Bowen EJ, Schmidt TW, Firm CS, Russo AF, Durham PL. Tumor necrosis factor-alpha stimulation of calcitonin gene-related peptide expression and secretion from rat trigeminal ganglion neurons. *J Neurochem.* 2006; 96(1):65–77. [PubMed: 16277606]
- Cady RJ, Denson JE, Sullivan LQ, Durham PL. Dual orexin receptor antagonist 12 inhibits expression of proteins in neurons and glia implicated in peripheral and central sensitization. *Neuroscience.* 2014; 269:79–92. [PubMed: 24685439]
- Cairns BE, Sessle BJ, Hu JW. Temporomandibular-evoked jaw muscle reflex: role of brain stem NMDA and non-NMDA receptors. *Neuroreport.* 2001; 12(9):1875–1878. [PubMed: 11435915]

- Capuano A, De Corato A, Lisi L, Tringali G, Navarra P, Dello Russo C. Proinflammatory-activated trigeminal satellite cells promote neuronal sensitization: relevance for migraine pathology. *Mol Pain*. 2009; 5:43. [PubMed: 19660121]
- Cavaillon JM. Pro-versus anti-inflammatory cytokines: myth or reality. *Cell Mol Biol (Noisy-le-grand)*. 2001; 47(4):695–702. [PubMed: 11502077]
- Cheng JK, Ji RR. Intracellular signaling in primary sensory neurons and persistent pain. *Neurochem Res*. 2008; 33(10):1970–1978. [PubMed: 18427980]
- Croll SD, Ransohoff RM, Cai N, Zhang Q, Martin FJ, Wei T, ... Wiegand SJ. VEGF-mediated inflammation precedes angiogenesis in adult brain. *Exp Neurol*. 2004; 187(2):388–402. [PubMed: 15144865]
- De Corato A, Lisi L, Capuano A, Tringali G, Tramutola A, Navarra P, Dello Russo C. Trigeminal satellite cells express functional calcitonin gene-related peptide receptors, whose activation enhances interleukin-1beta pro-inflammatory effects. *J Neuroimmunol*. 2011; 237(1–2):39–46. [PubMed: 21719118]
- Dinarello CA. Proinflammatory cytokines. *Chest*. 2000; 118(2):503–508. [PubMed: 10936147]
- Durham PL, Garrett FG. Emerging importance of neuron-satellite glia interactions within trigeminal ganglia in craniofacial pain. *TOPAINJ*. 2010; 3:3–13.
- Emshoff R, Puffer P, Rudisch A, Gassner R. Temporomandibular joint pain: relationship to internal derangement type, osteoarthritis, and synovial fluid mediator level of tumor necrosis factor-alpha. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. 2000; 90(4):442–449. [PubMed: 11027380]
- Fredriksson L, Alstergren P, Kopp S. Tumor necrosis factor-alpha in temporomandibular joint synovial fluid predicts treatment effects on pain by intra-articular glucocorticoid treatment. *Mediators Inflamm*. 2006; 2006(6):59425. [PubMed: 17392588]
- Fu K, Ma X, Zhang Z, Chen W. Tumor necrosis factor in synovial fluid of patients with temporomandibular disorders. *J Oral Maxillofac Surg*. 1995; 53(4):424–426. [PubMed: 7699497]
- Garrett FG, Hawkins JL, Overmyer AE, Hayden JB, Durham PL. Validation of a novel rat-holding device for studying heat- and mechanical-evoked trigeminal nocifensive behavioral responses. *J Orofac Pain*. 2012; 26(4):337–344. [PubMed: 23110274]
- Greenspan JD, Slade GD, Bair E, Dubner R, Fillingim RB, Ohrbach R, ... Maixner W. Pain sensitivity risk factors for chronic TMD: descriptive data and empirically identified domains from the OPPERA case control study. *J Pain*. 2011a; 12(11 Suppl):T61–74. [PubMed: 22074753]
- Greenspan JD, Slade GD, Bair E, Dubner R, Fillingim RB, Ohrbach R, ... Maixner W. Pain sensitivity risk factors for chronic TMD: descriptive data and empirically identified domains from the OPPERA case control study. *The journal of pain : official journal of the American Pain Society*. 2011b; 12(11 Suppl):T61–74. [PubMed: 22074753]
- Hanada T, Yoshimura A. Regulation of cytokine signaling and inflammation. *Cytokine Growth Factor Rev*. 2002; 13(4–5):413–421. [PubMed: 12220554]
- Hawkins JL, Denson JE, Miley DR, Durham PL. Nicotine stimulates expression of proteins implicated in peripheral and central sensitization. *Neuroscience*. 2015; 290:115–125. [PubMed: 25637801]
- Imbe H, Iwata K, Zhou QQ, Zou S, Dubner R, Ren K. Orofacial deep and cutaneous tissue inflammation and trigeminal neuronal activation. Implications for persistent temporomandibular pain. *Cells Tissues Organs*. 2001; 169(3):238–247. [PubMed: 11455119]
- Kacena MA, Merrel GA, Konda SR, Wilson KM, Xi Y, Horowitz MC. Inflammation and bony changes at the temporomandibular joint. *Cells Tissues Organs*. 2001; 169(3):257–264. [PubMed: 11455121]
- Kasama T, Miwa Y, Isozaki T, Odai T, Adachi M, Kunkel SL. Neutrophil-derived cytokines: potential therapeutic targets in inflammation. *Curr Drug Targets Inflamm Allergy*. 2005; 4(3):273–279. [PubMed: 16101533]
- Kristensen KD, Stoustrup P, Kuseler A, Pedersen TK, Nyengaard JR, Hauge E, Herlin T. Intra-articular vs. systemic administration of etanercept in antigen-induced arthritis in the temporomandibular point. Part I: histological effects. *Pediatr Rheumatol Online J*. 2009; 7:5. [PubMed: 19200377]
- Leung L, Cahill CM. TNF-alpha and neuropathic pain--a review. *J Neuroinflammation*. 2010; 7:27. [PubMed: 20398373]

- Linker RA, Maurer M, Gaupp S, Martini R, Holtmann B, Giess R, ... Gold R. CNTF is a major protective factor in demyelinating CNS disease: a neurotrophic cytokine as modulator in neuroinflammation. *Nat Med*. 2002; 8(6):620–624. [PubMed: 12042814]
- Lobbezoo F, Drangsholt M, Peck C, Sato H, Kopp S, Svensson P. Topical review: new insights into the pathology and diagnosis of disorders of the temporomandibular joint. *J Orofac Pain*. 2004; 18(3): 181–191. [PubMed: 15508997]
- Manfredini D, Guarda-Nardini L, Winocur E, Piccotti F, Ahlberg J, Lobbezoo F. Research diagnostic criteria for temporomandibular disorders: a systematic review of axis I epidemiologic findings. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. 2011; 112(4):453–462. [PubMed: 21835653]
- Mujakperuo HR, Watson M, Morrison R, Macfarlane TV. Pharmacological interventions for pain in patients with temporomandibular disorders. *Cochrane Database Syst Rev*. 2010; (10):CD004715. [PubMed: 20927737]
- Neeb L, Hellen P, Boehnke C, Hoffmann J, Schuh-Hofer S, Dirnagl U, Reuter U. IL-1beta stimulates COX-2 dependent PGE(2) synthesis and CGRP release in rat trigeminal ganglia cells. *PLoS One*. 2011; 6(3):e17360. [PubMed: 21394197]
- Nishimori T, Sera M, Suemune S, Yoshida A, Tsuru K, Tsuiki Y, ... Shigenaga Y. The distribution of muscle primary afferents from the masseter nerve to the trigeminal sensory nuclei. *Brain Res*. 1986; 372(2):375–381. [PubMed: 3486695]
- Nishimura M, Segami N, Kaneyama K, Suzuki T, Miyamaru M. Proinflammatory cytokines and arthroscopic findings of patients with internal derangement and osteoarthritis of the temporomandibular joint. *Br J Oral Maxillofac Surg*. 2002; 40(1):68–71. [PubMed: 11883975]
- Nordahl S, Alstergren P, Kopp S. Tumor necrosis factor-alpha in synovial fluid and plasma from patients with chronic connective tissue disease and its relation to temporomandibular joint pain. *J Oral Maxillofac Surg*. 2000; 58(5):525–530. [PubMed: 10800908]
- Ohtani T, Habu M, Khanal A, Yoshioka I, Matsukawa A, Tominaga K. Local effects of intra-articular injection of anti-rabbit tumor necrosis factor alpha monoclonal antibody in antigen-induced arthritis of the rabbit temporomandibular joint. *J Oral Pathol Med*. 2012; 41(1):96–105. [PubMed: 22004078]
- Palladino MA, Bahjat FR, Theodorakis EA, Moldawer LL. Anti-TNF-alpha therapies: the next generation. *Nat Rev Drug Discov*. 2003; 2(9):736–746. [PubMed: 12951580]
- Poveda Roda R, Bagan JV, Diaz Fernandez JM, Hernandez Bazan S, Jimenez Soriano Y. Review of temporomandibular joint pathology. Part I: classification, epidemiology and risk factors. *Med Oral Patol Oral Cir Bucal*. 2007; 12(4):E292–298. [PubMed: 17664915]
- Ren K, Dubner R. Neuron-glia crosstalk gets serious: role in pain hypersensitivity. *Curr Opin Anaesthesiol*. 2008; 21(5):570–579. [PubMed: 18784481]
- Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods*. 2012; 9(7):671–675. [PubMed: 22930834]
- Sessle BJ. Peripheral and central mechanisms of orofacial inflammatory pain. *Int Rev Neurobiol*. 2011; 97:179–206. [PubMed: 21708311]
- Shafer DM, Assael L, White LB, Rossomando EF. Tumor necrosis factor-alpha as a biochemical marker of pain and outcome in temporomandibular joints with internal derangements. *J Oral Maxillofac Surg*. 1994; 52(8):786–791. discussion 791–782. [PubMed: 8040729]
- Shankland WE 2nd. The trigeminal nerve. Part I: An over-view. *Cranio*. 2000; 18(4):238–248. [PubMed: 11202843]
- Shigenaga Y, Sera M, Nishimori T, Suemune S, Nishimura M, Yoshida A, Tsuru K. The central projection of masticatory afferent fibers to the trigeminal sensory nuclear complex and upper cervical spinal cord. *J Comp Neurol*. 1988; 268(4):489–507. [PubMed: 2451684]
- Slade GD, Bair E, By K, Mulkey F, Baraian C, Rothwell R, ... Ohrbach R. Study methods, recruitment, sociodemographic findings, and demographic representativeness in the OPERA study. *J Pain*. 2011; 12(11 Suppl):T12–26. [PubMed: 22074749]
- Song DD, Li Y, Tang D, Huang LY, Yuan YZ. Neuron-glia communication mediated by TNF-alpha and glial activation in dorsal root ganglia in visceral inflammatory hypersensitivity. *Am J Physiol Gastrointest Liver Physiol*. 2014; 306(9):G788–795. [PubMed: 24627565]

- Souza GR, Talbot J, Lotufo CM, Cunha FQ, Cunha TM, Ferreira SH. Fractalkine mediates inflammatory pain through activation of satellite glial cells. *Proc Natl Acad Sci U S A*. 2013; 110(27):11193–11198. [PubMed: 23776243]
- Spears R, Dees LA, Sapozhnikov M, Bellinger LL, Hutchins B. Temporal changes in inflammatory mediator concentrations in an adjuvant model of temporomandibular joint inflammation. *J Orofac Pain*. 2005; 19(1):34–40. [PubMed: 15779537]
- Stoustrup P, Kristensen KD, Kuseler A, Pedersen TK, Gelineck J, Herlin T. Intra-articular vs. systemic administration of etanercept in antigen-induced arthritis in the temporomandibular joint. Part II: mandibular growth. *Pediatr Rheumatol Online J*. 2009; 7:6. [PubMed: 19200378]
- Suzuki T, Segami N, Nishimura M, Nojima T. Co-expression of interleukin-1beta and tumor necrosis factor alpha in synovial tissues and synovial fluids of temporomandibular joint with internal derangement: comparison with histological grading of synovial inflammation. *J Oral Pathol Med*. 2002; 31(9):549–557. [PubMed: 12269994]
- Takeda M, Tanimoto T, Kadoi J, Nasu M, Takahashi M, Kitagawa J, Matsumoto S. Enhanced excitability of nociceptive trigeminal ganglion neurons by satellite glial cytokine following peripheral inflammation. *Pain*. 2007; 129(1–2):155–166. [PubMed: 17127002]
- Taylor PC, Williams RO, Feldmann M. Tumour necrosis factor alpha as a therapeutic target for immune-mediated inflammatory diseases. *Curr Opin Biotechnol*. 2004; 15(6):557–563. [PubMed: 15560982]
- Thalakoti S, Patil VV, Damodaram S, Vause CV, Langford LE, Freeman SE, Durham PL. Neuron-glia signaling in trigeminal ganglion: implications for migraine pathology. *Headache*. 2007; 47(7):1008–1023. [PubMed: 17635592]
- Villa G, Ceruti S, Zanardelli M, Magni G, Jasmin L, Ohara PT, Abbracchio MP. Temporomandibular joint inflammation activates glial and immune cells in both the trigeminal ganglia and in the spinal trigeminal nucleus. *Mol Pain*. 2010; 6:89. [PubMed: 21143950]
- Yan J, Melemedjian OK, Price TJ, Dussor G. Sensitization of dural afferents underlies migraine-related behavior following meningeal application of interleukin-6 (IL-6). *Mol Pain*. 2012; 8:6. [PubMed: 22273495]
- Zhang X, Burstein R, Levy D. Local action of the proinflammatory cytokines IL-1beta and IL-6 on intracranial meningeal nociceptors. *Cephalalgia*. 2012; 32(1):66–72. [PubMed: 22144718]

Highlights

- Injection of TNF- α in TMJ increases nocifensive response to mechanical stimulation
- TNF- α causes increase in level of multiple cytokines in trigeminal ganglion
- Increased nocifensive response correlated with differential cytokine regulation

Figure 1.

Bilateral injection of TNF- α into the TMJ capsule causes a transient increase in nocifensive response to mechanical stimulation. The average number of withdrawal responses in the cutaneous tissue over the TMJ at 2 and 24 hours to mechanical stimulation using a 100 g von Frey filament as compared to naïve and vehicle control is shown. The average number of withdrawal responses out of 5 for each condition is shown for naïve controls (blue), animals injected bilaterally with the vehicle PBS (red), and animals injected bilaterally with 100 ng/mL TNF- α (green). Animals that withdrew at least 3 out of 5 times to the filament were considered to have a positive and nocifensive response. Conditions in which average withdrawal responses were significantly higher than either basal or vehicle controls at $P < 0.05$ are denoted by #. Error bars indicate SEM, $n = 6$ per condition.

Figure 2.

Injection of PBS into both TMJ capsules increases expression of several cytokines in the trigeminal ganglion as compared to naïve control levels. The relative fold change in cytokine levels when compared to the average of naïve control animals that was set at a value of 1 is shown. Conditions in which protein expression is increased by PBS treatment relative to naïve control at $P < 0.05$ are denoted by *. Error bars indicate SEM, $n = 3$ per condition.

Figure 3.

Bilateral injection of TNF- α into TMJ capsule induces differential cytokine expression in the trigeminal ganglion as compared to naïve control levels. The relative fold change in TNF- α regulated cytokine levels at 2 hours (red) and 24 hours (green) post injection when compared to the average of naïve control animals that was set at a value of 1 is shown. Conditions in which protein expression is increased by TNF- α treatment at 2 or 24 hours relative to naïve control at $P < 0.05$ are denoted by *. Error bars indicate SEM, $n = 3$ per condition.

Figure 4.

TNF- α injection into both TMJ capsules increases the level of several cytokines in the trigeminal ganglion as compared to values for vehicle treated animals. The relative fold change in TNF- α regulated cytokine levels at 2 hours (red) and 24 hours (green) post injection when compared to the average of vehicle treated animals that was set at a value of 1 is shown. Conditions in which protein expression is increased by TNF- α treatment at 2 or 24 hours relative to vehicle control at $P < 0.05$ are denoted by *. Error bars indicate SEM, $n = 3$ per condition.

Table 1

Fold Change In Cytokine Levels Compared to Naïve Control $\pm$ SEM. The average relative intensity value for the naïve control animals was set equal to 1.

Protein	Vehicle Control	TNF α 2hr	TNF α 24hr
CINC-1	2.40 $\pm$ 0.47	4.13 $\pm$ 0.58	0.78 $\pm$ 0.23
CINC-2 α/β	2.35 $\pm$ 0.31	2.33 $\pm$ 0.25	3.87 $\pm$ 1.94
CNTF	0.61 $\pm$ 0.12	1.39 $\pm$ 0.14	1.23 $\pm$ 0.13
Fractalkine	2.82 $\pm$ 0.43	6.60 $\pm$ 1.24	2.92 $\pm$ 0.37
GM-CSF	3.26 $\pm$ 0.54	6.32 $\pm$ 1.54	2.84 $\pm$ 0.67
sICAM-1	1.16 $\pm$ 0.09	1.79 $\pm$ 0.39	1.07 $\pm$ 0.22
IFN- γ	2.36 $\pm$ 0.18	2.60 $\pm$ 0.32	1.45 $\pm$ 0.23
IL-1 α	2.81 $\pm$ 0.24	5.07 $\pm$ 0.77	2.18 $\pm$ 0.20
IL-1 β	1.90 $\pm$ 0.26	3.97 $\pm$ 0.28	1.42 $\pm$ 0.40
IL-3	4.84 $\pm$ 1.37	8.65 $\pm$ 1.89	4.30 $\pm$ 0.96
IL-4	4.25 $\pm$ 1.24	7.79 $\pm$ 2.13	3.18 $\pm$ 0.76
IL-6	2.66 $\pm$ 0.12	5.17 $\pm$ 1.07	1.56 $\pm$ 0.07
IL-10	1.93 $\pm$ 0.07	2.57 $\pm$ 0.25	1.56 $\pm$ 0.17
IP-10	3.71 $\pm$ 0.68	7.83 $\pm$ 1.29	2.95 $\pm$ 1.03
LIX	2.09 $\pm$ 0.69	3.90 $\pm$ 0.16	1.86 $\pm$ 0.39
L-Selectin	2.63 $\pm$ 0.62	7.90 $\pm$ 3.68	1.55 $\pm$ 0.44
MIG	4.02 $\pm$ 1.06	6.33 $\pm$ 0.38	2.29 $\pm$ 0.33
MIP-1 α	2.57 $\pm$ 0.28	3.37 $\pm$ 0.65	2.02 $\pm$ 0.23
MIP-3 α	2.08 $\pm$ 0.15	2.97 $\pm$ 0.33	1.20 $\pm$ 0.11
Thymus Chemokines	0.66 $\pm$ 0.06	0.96 $\pm$ 0.05	1.00 $\pm$ 0.06
TIMP-1	1.48 $\pm$ 0.15	5.19 $\pm$ 2.37	0.46 $\pm$ 0.13
VEGF	2.87 $\pm$ 0.68	5.10 $\pm$ 0.49	2.10 $\pm$ 0.10

Table 2

Fold Change In Cytokine Levels Compared to Vehicle Control $\pm$ SEM. The average relative intensity value for the vehicle treated animals was set equal to 1.

Protein	TNF α 2hr	TNF α 24hr
CINC-1	2.08 $\pm$ 0.48	0.35 $\pm$ 0.08
CINC-2 α/β	1.01 $\pm$ 0.07	1.50 $\pm$ 0.59
CNTF	2.67 $\pm$ 0.43	2.48 $\pm$ 0.50
Fractalkine	2.34 $\pm$ 0.23	1.24 $\pm$ 0.32
GM-CSF	1.80 $\pm$ 0.17	0.87 $\pm$ 0.10
sICAM-1	1.48 $\pm$ 0.24	0.90 $\pm$ 0.14
IFN- γ	1.15 $\pm$ 0.19	0.62 $\pm$ 0.10
IL-1 α	1.78 $\pm$ 0.22	0.77 $\pm$ 0.04
IL-1 β	2.18 $\pm$ 0.17	0.69 $\pm$ 0.14
IL-3	3.08 $\pm$ 1.01	1.37 $\pm$ 0.36
IL-4	2.36 $\pm$ 0.45	1.11 $\pm$ 0.29
IL-6	1.98 $\pm$ 0.43	0.59 $\pm$ 0.03
IL-10	1.33 $\pm$ 0.11	0.80 $\pm$ 0.07
IP-10	2.34 $\pm$ 0.44	1.17 $\pm$ 0.52
LIX	3.89 $\pm$ 1.50	1.31 $\pm$ 0.28
L-Selectin	2.28 $\pm$ 0.65	0.56 $\pm$ 0.69
MIG	2.79 $\pm$ 0.97	0.84 $\pm$ 0.22
MIP-1 α	1.27 $\pm$ 0.13	0.80 $\pm$ 0.08
MIP-3 α	1.41 $\pm$ 0.11	0.58 $\pm$ 0.04
Thymus Chemokines	1.55 $\pm$ 0.21	1.58 $\pm$ 0.16
TIMP-1	3.43 $\pm$ 1.47	0.30 $\pm$ 0.08