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Hippocampal Nerve Growth Factor Potentiated by 17β-Estradiol and Involved in Allodynia of Inflamed TMJ in Rat

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Abstract: The hippocampus is believed to play an important role in sex-based differences of pain perception. Whether estrogen potentiates allodynia in the inflamed temporomandibular joint (TMJ) through affecting the expressions of pain-related genes in the hippocampus remains largely unknown. Because the nerve growth factor (NGF) is an important gene related to inflammatory pain, we tested whether hippocampal NGF may be involved in TMJ inflammatory pain. Here we showed that the rat hippocampal NGF was upregulated by TMJ inflammation induced by complete Freund adjuvant. NGF upregulation was further potentiated by estradiol in a dose-dependent manner. In contrast, NGF transcription in the amygdala, prefrontal cortex, and thalamus was not affected by TMJ inflammation and estradiol. An intrahippocampal injection of NGF antibody or NGF receptor inhibitor K252a (inhibitor for tropomyosin receptor kinase A, TrkA) reduced the allodynia of inflamed TMJ in proestrous rats. Our data suggest that the hippocampal NGF is involved in estradiol-sensitized allodynia of inflammatory TMJ pain.

***Perspective:** We report that complete Freund adjuvant–induced temporomandibular joint (TMJ) inflammation upregulated hippocampal nerve growth factor (NGF) expression, and estradiol replacement potentiated this upregulation. These results propose that estradiol could modulate TMJ pain through the NGF signaling pathway in the hippocampus to exacerbate TMJ pain and offer a possible mechanism of sexual dimorphism of temporomandibular disorder pain.*

***Key words:** Estrogen, nerve growth factor (NGF), temporomandibular disorder (TMD), pain, hippocampus.*

Temporomandibular disorder (TMD)-related pain includes myofascial masticatory muscle pain and temporomandibular joint (TMJ) pain. Joint inflammation is believed to be the major reason for TMJ pain.[32,33,37,46] TMD has the highest prevalence in women aged 20 to 40, approximately twice that found in men.[67] Although the mechanism underlying the sex-based difference of TMD pain remains unclear, the central hippocampus is believed to play an important role in the sexual dimorphism of pain syndromes.[2,3,31] We recently reported that estradiol can potentiate the expression of hippocampal transient receptor potential vanilloid 1 (TRPV1) and enhance the allodynia of an inflamed TMJ in female rats. Intrahippocampal injection of TRPV1 antagonists partially blocks estradiol-enhanced allodynia of the inflamed TMJ, suggesting that estradiol enhances the allodynia of the inflamed TMJ at least partially through hippocampal TRPV1.[70] However, nerve growth factor (NGF) is an important regulator of TRPV1, which can regulate TRPV1 sensitization, translocation, and transcription,[71,73] and NGF is particularly involved in inflammatory pain.[4] Therefore, we question whether the hippocampal NGF was also involved in TMJ inflammatory pain in female rats.

NGF is essential for the development and maintenance of the central nervous system (CNS) and peripheral nervous system (PNS).[17] NGF is secreted as a precursor and cleaved by its regulators to generate mature NGF.[13] Both NGF and its receptors are expressed and functional

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in the hippocampus.[7,10,16,56,66] Hippocampal NGF expression is induced by a variety of stimuli, including hypothermia, memory task, traumatic brain injury, transient cerebral ischemia, early maternal separation, and even nicotine.[10,20,28,59,69] Three decades ago, NGF was also found to be inflammation induced and to play an important role in inflammatory pain in male rats.[68] NGF acts as a pain-related gene function through 2 distinct types of high-affinity cell-surface receptors, tropomyosin receptor kinase A (TrkA) receptor and p75; TrkA appears to be more important than p75 in inflammatory pain in both sexes of mice.[6] The question then arises whether TMJ inflammation and estrogen affect hippocampal NGF expression, and whether hippocampal NGF is involved in the allodynia of TMJ inflammation in female rats. To address this question, we examined the hippocampal NGF expression in ovariectomized female rats with inflamed TMJ to test whether 17β-estradiol could affect NGF expression and whether intrahippocampal injection of the NGF antibody or NGF TrKA antagonist could have any effect on the allodynia of inflamed TMJ.

Methods

Animals

Two weight classes of Sprague-Dawley female rats were used (Vital River Laboratory Animal Technology Co. Ltd, Beijing, China). The experiment was approved by the Animal Use and Care Committee of Peking University and was consistent with the Ethical Guidelines of the International Association for the Study of Pain. Female rats weighing 180 to 200 g were ovariectomized, received estradiol replacement, and were randomly divided into 6 groups (6 rats per group), including 1 control group, 1 sham-ovariectomized group, and 4 groups of ovariectomized rats dosed at 0, 20, 80, or 200 μg of 17β-estradiol, respectively. Female rats weighing 240 to 260 g received the cannula placement and were randomly divided into 6 groups (5 rats per group), including 2 groups receiving NGF antibody (2 or 20 ng), 2 groups receiving K252a (5 or 25 ng), and 2 groups receiving vehicles as the NGF antibody or K252a controls, respectively.

Estradiol Administration

The rats were anesthetized by intraperitoneal injections of pentobarbital sodium (50 mg/kg body weight). Following anesthesia induction, the rats were bilaterally ovariectomized or sham-operated (control and sham-ovariectomized groups) and allowed to recover for 1 week. 17β-estradiol (Beijing Huanmeihuli Biochemical, Beijing, China) was dissolved in corn oil. The ovariectomized rats received 17β-estradiol by subcutaneous injection every morning for 12 days. The doses were 0, 20, 80, or 200 μg per rat, respectively, with a total dosage volume of 200 μL. The control, sham-ovariectomized, and ovariectomized rats that received 0 μg estradiol were subcutaneously injected with an equal amount of corn oil. Vaginal smears confirmed the estrous stages in the control and sham-ovariectomized groups.

Induction of TMJ Inflammation

On the 12th day of estradiol replacement, the sham-ovariectomized and ovariectomized rats were anesthetized and injected with 50 μL of complete Freund's adjuvant (CFA) (1:1 oil/saline suspension) (Sigma-Aldrich, St. Louis, MO) into the bilateral TMJs to induce TMJ inflammation for 24 hours as described previously.[55] Briefly, the injection site was positioned inferior to the posteroinferior margin of the zygomatic arch. A 30-gauge needle was fixed anteriorly until the needle contacted the posterolateral aspect of the condyle. CFA was injected following a gentle aspiration. The control rats were injected with 50 μL of saline into the bilateral TMJs.

Measurement of Head Withdrawal Threshold

The head withdrawal threshold is negatively associated with mechanical sensitivity of the orofacial region as reported previously.[55,70] The head withdrawal threshold was measured before and after CFA injection. The rats were trained to rear on their hind paws and recline against the experimenter's working glove. Although the rats could move freely, they kept motionless during the test session. The increasing forces from the electronic von Frey filament (IITC Life Science, Woodland Hills, CA) was introduced to the TMJ region until the head was withdrawn and the applied force was recorded. The head withdrawal threshold was calculated as mean ± standard error of the mean (SEM) based on at least 5 times per joint and 6 rats per group.

Measurement of Food Intake

Food intake is also negatively associated with TMJ inflammation/pain and used as an indicator for TMJ inflammation/pain.[30,36] Food intake was measured before and after CFA injection for each rat. Briefly, each rat was isolated with water only for 20 hours. The food was weighed and given to the rat for 2 hours without water, to exclude the effect of drinking. Afterwards, the amount of uneaten food was weighed and the amount eaten was calculated.

Measurement of Circulating Estradiol

The rats were sacrificed with an overdose of sodium pentobarbital (100 mg/kg body weight) 24 hours after induction of TMJ inflammation, and blood was collected from the inferior vena cava. Circulating estradiol was measured when each rat was in the same phase of the estrous cycle. All 6 groups (6 rats per group) were measured by radioimmunoassay using commercially available ^{125}I radioimmunoassay kit (17β-estradiol double antibody, Beijing North Institute of Biological Technology, Beijing, China) and an Access Immunoassay System (Beckman Coulter, Brea, CA).

Verification of CFA-Induced TMJ Inflammation

CFA-induced TMJ inflammation was examined as described in our previous study.[70] Briefly, whole TMJs

were dissected from the rats and fixed in 4% paraformaldehyde in .1M phosphate buffered saline (PBS) and demineralized in 15% ethylenediaminetetraacetic acid (EDTA). The specimens were processed with graded alcohols and xylene, embedded in paraffin, sagittally sectioned at 5 μm, and stained with hematoxylin and eosin.

Quantitative Real-Time Polymerase Chain Reaction

The whole hippocampus, amygdala, prefrontal cortex, and thalamus were bilaterally harvested from 3 rats per group (including control group, 1 sham-ovariectomized group, and 4 groups of ovariectomized rats dosed at 0, 20, 80, or 200 μg of 17β-estradiol) and homogenized by a homogenizer (Ultra-Turrax T10; IKA Lab Technology, Staufen, Germany) in ice-cold TRIzol (Invitrogen, Carlsbad, CA) for RNA extraction. Total RNA was extracted with TRIzol in accordance with the manufacturer's instructions and the integrity was evaluated by electrophoresis in 1% agarose gel. The real-time polymerase chain reaction (RT-PCR) was conducted with the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) in a 20-μL reaction volume containing 1 μg of RNA at 42°C for 30 minutes, and terminated by heating at 85°C for 5 minutes. The synthesized cDNA was stored at −20°C until use.

RT-PCR was performed with Power SYBRGreen PCR Master Mix (Applied Biosystems, Foster City, CA) using the 7500 Real-Time PCR System (Applied Biosystems). The reactions were run in duplicate with 1 μL of cDNA template in a 20-μL reaction volume; the program operated at 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 94°C for 15 seconds and 60°C for 1 minute. The amplification specificity was confirmed by a melting curve. The mRNA level of the target gene was acquired from the threshold cycle value relative to that of β-actin through the formula $2^{-\Delta Ct}$ (ΔCt = β-actin Ct – gene of interest Ct).[26] The efficiency of the primers was confirmed by sequencing the conventional PCR products before applying RT-PCR. The following primers were synthesized according to sequences specified in previous reports: rat NGF sense/antisense, 5′-TAAGAGTACCCACAAAGTTT-3′/5′- CCTGCTTTCTGACCAGTCTT-3′[72]; rat β-actin sense/antisense, 5′-TGACAGGATGCAGAAGGAGA-3′/5′-TAGAGCCACCAATCCACACA-3′.[65]

Western Blot Analysis

The whole hippocampus was removed from 2 rats per group, separate from the RT-PCR group, and homogenized by the homogenizer in ice-cold denaturing lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, 1 μg/mL leupeptin), then centrifuged at 13,000 *g* for 20 minutes at 4°C. The supernatant was collected and protein concentrations were determined using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). Protein samples were subjected to 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane. The membrane was blocked in 5% nonfat dry milk in TBS-T buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, .05% Tween-20) for 1 hour at room temperature and probed with primary anti-NGF antibodies (SC-548, H-20; Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:1000 overnight at 4°C.[8,9] The membrane was washed extensively with TBS-T and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature. After the extensive wash with TBS-T, the membrane was visualized using the ECL kit (Applygen Technologies Inc., Beijing, China). For internal control, the blots were stripped and reprobed with anti-β-actin antibodies (Santa Cruz Biotechnology) at a dilution of 1:1000.

Intrahippocampal Injection of NGF Antibody or NGF Antagonist into CA1 Region of Hippocampus

TrKA antagonist K252a (Sigma-Aldrich) was dissolved in dimethylsulfoxide (DMSO). The doses of K252a (5 and 25 ng in 1 μL) and NGF antibody (2 and 20 ng in 1 μL, diluted in PBS) were used based on previous studies,[35,50,53] in which injection of NGF antibody or K252a into the amygdala or red nucleus showed anti-conditioned fear or analgesic effects. The solutions were prepared immediately before use.

Female rats (240–260 g) were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneal injection). Guide cannulas (5 mm in length and .6 mm in outer diameter, .3 mm in inner diameter) were bilaterally inserted until 1 mm above the CA1 region of the hippocampus according to the following parameters: anteroposterior = −3.5 mm, left to right = ± 2.4 mm (10°angle), and dorsoventral = −2 mm relative to the bregma and skull surface.[51] The cannulas were anchored to the skull with stainless steel screws and dental self-curing acrylic resin. A stainless steel blocker was inserted into the cannula to prevent obstruction and infection. The rats were allowed to recover from surgery for at least 7 days. Estrous stages were determined by vaginal smear (Supplemental Fig 1). Following the vaginal smear, late-metestrus-stage rats received a first intrahippocampal injection after the CFA injection as previously reported.[70]

Intrahippocampal injections of NGF antibody or K252a into the bilateral CA1 regions of the hippocampi were performed with a micromanipulator mounted with two 1-μL Hamilton microsyringes (Hamilton, Reno, NV). Each microsyringe was connected through a PE-10 polyethylene catheter to an injection cannula that was introduced into the guide cannula with its tip extending 1 mm beyond the tip of the guide cannula. The bilateral hippocampi were simultaneously injected with 1 μL of the NGF antibody or K252a or vehicle over 1 minute, and the injection cannula was still kept in place for an additional 1 minute to allow for diffusion.

The effect of intrahippocampal injection of NGF antibody (2 and 20 ng, n = 5) and vehicle (PBS, n = 5) or K252a (5 and 25 ng, n = 5) and vehicle (saline/DMSO at ratio of 1:1, n = 5) on the baseline of head withdrawal threshold and food intake was first examined. The head

withdrawal threshold was measured for a period of 65 minutes after the hippocampal injection. Then, 8 hours later, the rats were injected with CFA into the TMJs to induce TMJ inflammation. Head withdrawal thresholds were measured 20 hours after CFA injection. The rats were immediately injected intrahippocampally with NGF antibody (2 and 20 ng) or K252a (5 and 25 ng), and head withdrawal thresholds were remeasured for a period of 65 minutes. During the 20-hour period of TMJ inflammation induction, the rats were supplied with water only; food intake was measured after the intrahippocampal injections and the head withdrawal threshold was measured. Behavioral testing was conducted on a double blind basis, in which the investigator did not know what was intrahippocampally injected into the rats. The rats were euthanized to examine placement of the cannulas.

Confirmation of Cannula Placement

Placement of the brain cannulas were examined as described previously.[70] Briefly, to verify cannula placement in the CA1 region, coronal sections (30 μm) of the brain were sliced on a cryostat, and the sections were examined under a microscope.

Statistical Analysis

Statistical analysis was performed with SPSS v.11.5 for Windows (SPSS Inc, Chicago, IL). All data were presented as mean ± SEM. The gene expression differences between the 6 groups were analyzed by two-way repeated measures ANOVA. The head withdrawal threshold and food intake at the same time point in the different groups were analyzed using independent-sample t test. A value of $P < .05$ was considered statistically significant.

Results

Effect of Estradiol Administration on Plasma Levels of Estradiol

To verify the effectiveness of the ovariectomy and estradiol administration, the plasma levels of estradiol were measured by radioimmunoassay, as shown in Table 1. The ovariectomized groups receiving the increasing doses of estradiol increased dose-dependently (n = 6, $P < .05$), with the 0- and 200-μg groups being expectedly the lowest and highest among the groups, respectively. The plasma levels of estradiol were similar in the control group (19.07 ± 3.34 pg/mL, n = 6), sham group (27.70 ± 7.60 pg/mL, n = 6), and 20-μg group (25.88 ± 8.4 pg/mL, n = 6), and they were higher than in the 0-μg group (12.26 ± 1.69 pg/mL) (n = 6, $P < .05$). The estradiol plasma levels of estradiol in the replaced groups were within the physiological range according to previous studies (additional information is available from the corresponding author).[38,70]

Upregulation of Hippocampal NGF by TMJ Inflammation and Further Potentiated by Estradiol

To explore whether the hippocampal NGF was involved in allodynia of inflamed TMJ and NGF further potentiated by estradiol, the expression of NGF in the hippocampus was first examined. As shown in Figs1 A and 1B, both mRNA and protein expressions of hippocampal NGF were induced in the TMJ inflammation groups compared to the control group ($P < .05$). However, this was not seen in the group dosed with 0 μg estradiol. The enhanced NGF expression in the hippocampus was further potentiated by estradiol in a dose-dependent manner in the ovariectomized groups. The expression of NGF protein in the hippocampus was mainly detected in the form of precursor NGF species (75, 60, 25, and 22 kDa) (Fig 1B), which corresponds to previous studies in which the NGF precursors were the predominant form of NGF in the human and rat brain.[13,23]

To examine whether the induction of NGF expression in the hippocampus was specific to the hippocampus, we also investigated the expression of NGF mRNA in the amygdala, prefrontal cortex, and thalamus. The expression of NGF mRNA in these areas was not affected by TMJ inflammation and estradiol replacement ($P < .05$) (Fig 2), suggesting that the expression of NGF mRNA in the hippocampus by TMJ inflammation and estradiol replacement was relatively specific.

Blocking NGF in Hippocampus Attenuated Mechanical Allodynia of Inflamed TMJ

To confirm whether the hippocampal NGF was involved in TMJ nociception, we performed an intrahippocampal injection of NGF antibody or NGF receptor antagonist K252a into the CA1 region to block NGF functions in the neurons of the CA1 region in vivo. The placements of all the cannulas were confirmed to be within the CA1 region between bregma −3.14 mm and −4.16 mm (Supplemental Fig 2). As shown in Fig 3, before the induction of TMJ inflammation, the baseline of head withdrawal threshold and food intake were not affected during the period of measurement by intrahippocampal injections of NGF antibody (2 and 20 ng) or K252a (5 and 25 ng) or vehicle into the CA1 region ($P < .05$). However, the head withdrawal threshold significantly decreased to about one-fifth of the baseline value after the

Table 1. Serum 17-Beta-Estradiol Level in Different Groups

			17-Beta-Estradiol Replacement Groups (μg)			
	Control	Sham	0	20	80	200
Serum estradiol (pg/mL)	19.07 ± 3.34	27.70 ± 7.60	12.26 ± 1.69	25.88 ± 8.43	53.93 ± 8.76	102 ± 20.52

NOTE. Data were presented as mean ± SEM, n = 6.

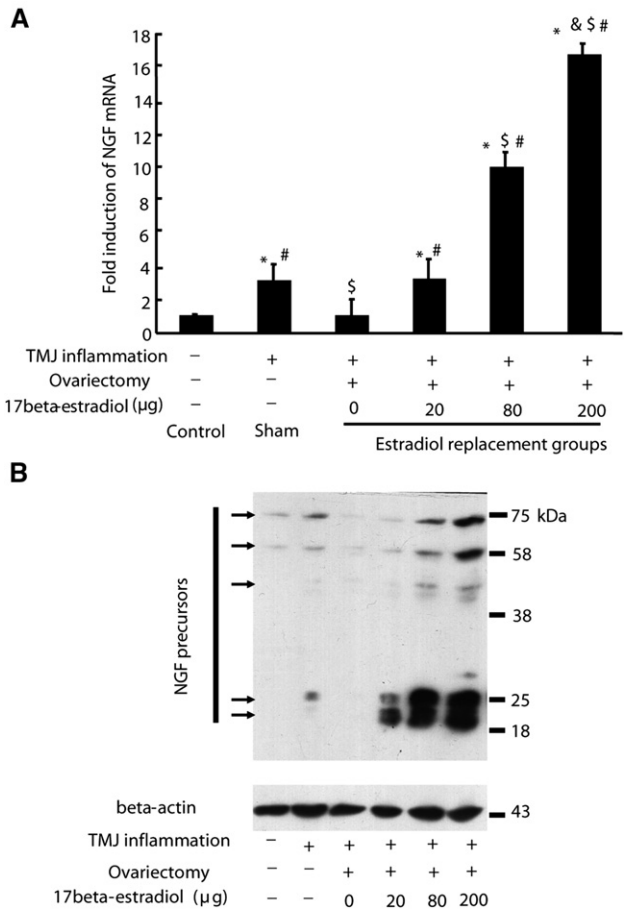

Figure 1. Hippocampal NGF expression was upregulated by TMJ inflammation and further potentiated by estradiol. **(A)** RT-PCR analysis for NGF expression in the hippocampus. The hippocampal NGF mRNA was induced by TMJ inflammation and further potentiated by estradiol in the ovariectomized rats. Note: TMJ inflammation failed to induce hippocampal NGF mRNA in the ovariectomized rats replaced with no estradiol. #$P < .05$ versus control group; *$P < .05$ versus 0-μg group; \$$P < .05$ versus 20-μg group; &$P < .05$ versus 80-μg group (n = 3, two-way ANOVA). **(B)** Representative immunoblotting for NGF expression in the hippocampus. β-actin served as an internal control for equal loading.

induction of TMJ inflammation. Although it was not statistically significant, intrahippocampal injection of 2 ng NGF antibody or 5 ng K252a partially counteracted the TMJ inflammation-induced decrease in head withdrawal threshold to some extent. Intrahippocampal injection of 20 ng NGF antibody or 25 ng K252a partially reversed TMJ inflammation-induced decrease in the head withdrawal thresholds at 2 time points (25 and 40 minutes) after NGF antibody microinjection and all the time points (5 , 15 , 25, 40, and 65 minutes) after K252a microinjection, respectively ($P < .05$) (Figs 3A and 3B). Intrahippocampal injections of NGF antibody or K252a also partially reversed TMJ inflammation-induced decrease in food intake (Figs 3C and 3D).

Discussion

Previous studies have explored that estradiol, which acts at both peripheral nociceptors and central processing

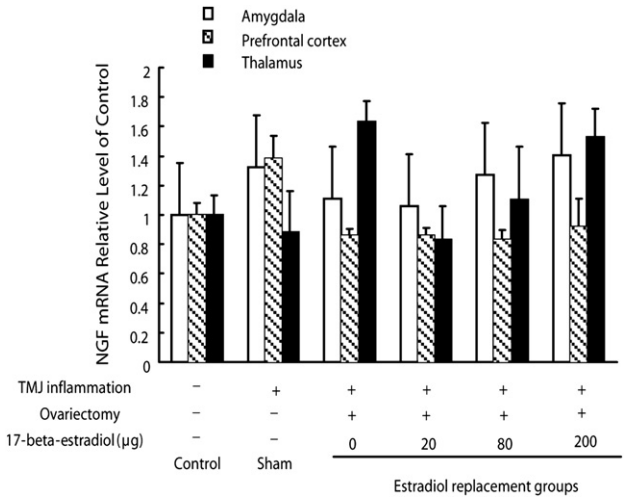

Figure 2. NGF mRNA expression in the amygdala, prefrontal cortex, and thalamus in rats was not affected by TMJ inflammation and estradiol replacement in the ovariectomized rats (n = 6, $P < .05$).

pathways,[5,13,22,24] is a possible modulator of TMJ pain sensitivity.[14,27,40] In this study, we showed 2 important findings: hippocampal NGF expression was upregulated by TMJ inflammation and further potentiated by estradiol in female rats, and blocking the hippocampal NGF pathway partially reversed the allodynia of inflamed TMJ. These results suggested that the hippocampal NGF was involved in central pain processing and could possibly be one of the molecular mechanisms underlying the sexually dimorphic TMD pain mediated by estradiol in the CNS.

Hippocampal NGF Involved in Sensitization of TMJ Nociception

In addition to its basic function as a neurotrophin for growth, maintenance, and survival of sympathetic and sensory neurons, NGF is also a pain-related gene involving inflammatory pain in both the CNS and PNS. In the PNS, injection of NGF into the masseter muscle of female rat evokes a long-lasting mechanical allodynia by increasing the sensitivity of peripheral nociceptors, and CFA-induced TMJ inflammation in male rats upregulates the NGF expression in TMJ tissues and trigeminal ganglion.[61,62] The administration of the TrkA receptor antagonist (K252a) could block NGF-induced TMJ spontaneous nociception in male rats, suggesting that NGF and TrkA play a functional role in TMJ inflammatory hyperalgesia.[52] In the CNS, both NGF and its receptor TrkA are also expressed in the hippocampus, and several types of stimuli induce their expressions. It is evident that central NGF may play an important role in broad-spectrum hyperalgesia. For instance, the concentration of NGF in cerebrospinal fluid increases in patients with fibromyalgia or chronic daily headaches,[27,57] and intracerebroventricular infusion of NGF induces a painlike response in male rats and patients with Alzheimer's disease.[22,29] The information about orofacial noxious stimuli can reach the hippocampus, and the hippocampus can trigger functional activity changes

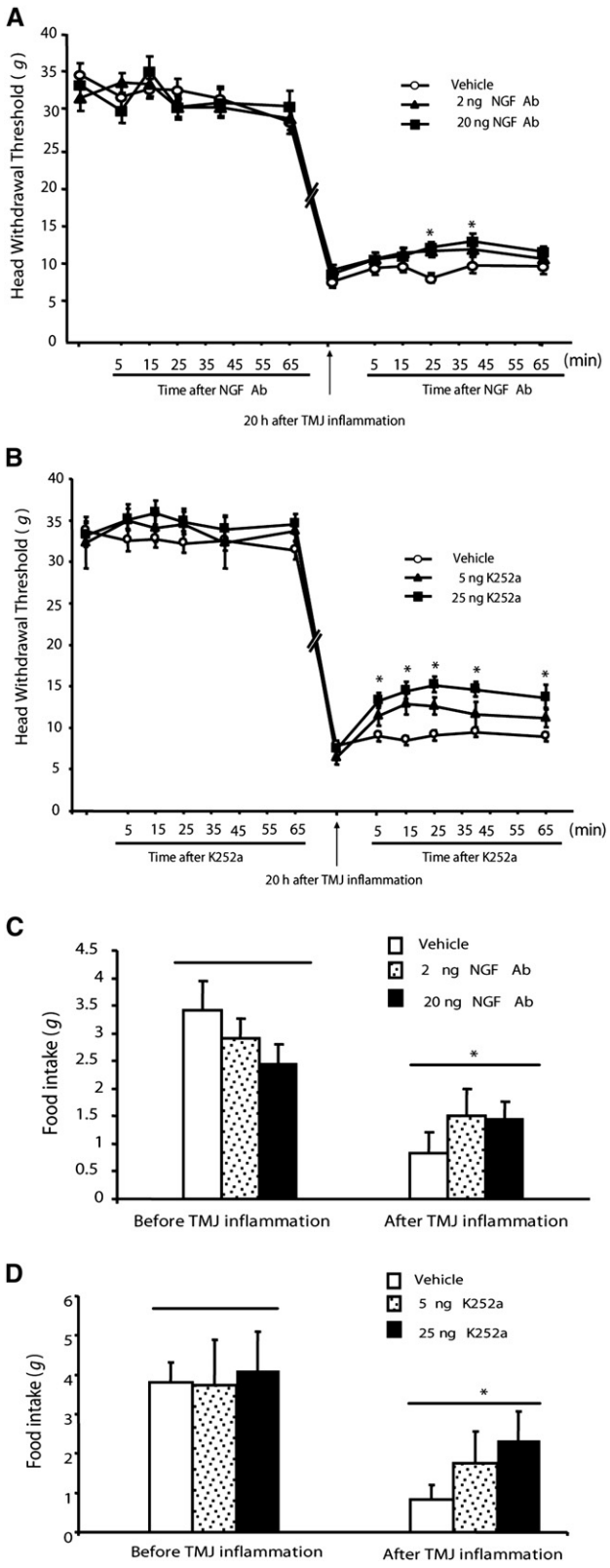

Figure 3. Attenuation of mechanical allodynia of inflamed TMJ by blocking NGF in the hippocampus. Head withdrawal threshold was partially reversed by intrahippocampal injections of NGF antibody (2 or 20 ng; n = 5) or vehicle (n = 5) **(A)** or K252a (5 or 25 ng, n = 5) or vehicle (n = 5) **(B)** into the CA1 region of the hippocampus. Prior to the induction of TMJ inflammation, the baseline head withdrawal threshold was not different between the intrahippocampal injections ($P < .05$). However, 20 hours after TMJ inflammation induction, the threshold dramatically decreased, and the decrease was partially reversed by intrahippocampal injection of NGF antibody (20 ng, n = 5) and K252a (25 ng, n = 5), but not by vehicle (n = 5), respectively. *$P < .05$ versus vehicle group at the same time point (independent-sample t test). Food intake improved after intrahippocampal injection of NGF antibody (2 or 20 ng, n = 5) **(C)** and NGF antagonist K252a (5 or 25 ng, n = 5) or vehicle (n = 5) **(D)** into the CA1 region. Prior to TMJ inflammation induction, food intake was not different between intrahippocampal injection of NGF antibody, or K252a. After TMJ inflammation induction, intrahippocampal injections of NGF antibody or K252a partially reversed the TMJ inflammation-induced decrease in food intake. *$P < .05$ versus the groups before induction of TMJ inflammation.

within the caudal trigeminal nucleus region.[12,39] However, whether the hippocampal NGF is also involved in orofacial inflammatory pain and sexual dimorphism of pain perception still needs to be defined. Our results showed that TMJ inflammation induced hippocampal NGF expression, and blocking the hippocampal NGF pathway alleviated inflamed TMJ allodynia, suggesting that the hippocampal NGF was involved in TMJ nociception. Therefore, the effect of TMJ inflammation on NGF expression in the hippocampus was relatively specific, but not due to the general effect of NGF expression in other brain regions such as in the amygdala, prefrontal cortex, and thalmus. Our results provided the first direct evidence on the involvement of hippocampal NGF in TMJ nociception. Given that estradiol further potentiated TMJ inflammation-induced upregulation of hippocampal NGF, and blocking the hippocampal NGF pathway partially reduced the allodynia of inflamed TMJ in rats at the proestrous stage, when rats have the highest amount of circulating estradiol in the estrous cycle stages, it would be further suggested that the hippocampal NGF was also involved in estradiol-enhanced TMJ nociception. Considering that NGF is an important regulator of TRPV1[71,73] and our previous observation that estradiol enhances TMJ nociception partially through induction of hippocampal TRPV1,[70] it is reasonably believed that the hippocampal NGF-TRPV1 signaling pathway would be involved in the estrogen-induced TMJ nociception in female rats. This could be an important central mechanism underlying the sexual dimorphism of TMD pain.

Central Mechanism Underlying Modulation of Gender-Based Difference in Inflammatory TMJ Perception by Estradiol

Several painful clinical conditions are more prominent in women and have been associated with the circulating serum level or fluctuation of the estradiol, such as clinical TMD pain,[40,41,43,67] migraines,[11,45] arthritis,[14,19,60] and fibromyalgia.[49] Many studies have been done to understand how estrogen affects peripheral and central nociceptive processes related to TMJ inflammatory pain.[5,15,24,25,47,48,64,70] However, whether estrogen enhances or decreases TMJ nociception remains controversial among these studies. Evidence shows that estrogen can sensitize TMJ nociception through central trigeminal ganglion.[5,48,64] Consistently, both our

present and previous results support this estrogen central sensitization of nociceptive processes and further reveal that estrogen could also enhance TMJ nociception through the hippocampal NGF-TRPV1 signaling pathway in rats.

Possible Downstream Targets of Hippocampal NGF That Are Involved in TMJ Nociception

TRPV1 is upregulated by NGF both in vitro and in vivo,[34,71,73] demonstrating that TRPV1 is one of the downstream targets for NGF. In addition, NGF-induced thermal hyperalgesia is not present in TRPV1 null mice, suggesting that TRPV1 is principally required for NGF-mediated thermal hyperalgesia.[18] The involvement of the hippocampal NGF in the allodynia of the inflamed TMJ may be through the induction of hippocampal TRPV1. However, the involvement of NGF in hyperalgesia may be also through the induction of peptide neurotransmitters, such as calcitonin gene-related peptide and substance P, as they are also downstream targets of NGF involved in hyperalgesia.[21,44]

Different Efficiencies of Intrahippocampal Injection of NGF Antibody or NGF Antagonist in Reducing Allodynia of Inflamed TMJ

The NGF receptor TrkA is more involved than p75 in inflammatory pain[6] and has demonstrated its functionality in the hippocampus.[1] Consistent with these observations, we observed that intrahippocampal injection of NGF antagonist K252a could partially reduce TMJ nociception. However, we also noticed that intrahippocampal injection of NGF antagonist K252a was more potent than NGF antibody in reducing the mechanical allodynia of the inflamed TMJ. This could be due to the fact that K252a was dissolved in DMSO, which has a strong ability to diffuse through the cytoplasmic membrane, whereas the NGF antibody diluted in PBS may have a lower ability to diffuse through the cytoplasmic membrane. Therefore, the solvents of the 2 substances may contribute, to some extent, to their different effects on reducing the TMJ nociception, despite the efficiency of the NGF antibody or antagonist used. In addition, K252a is a relatively potent NGF receptor blocker of the tyrosine protein kinase and is the most widely used TrkA receptor antagonist.[54,63] K252a inhibits not only NGF receptors but also other receptor tyrosine kinases[42,58] that may also play a role in the difference.

In conclusion, we have demonstrated that estradiol potentiated hippocampal NGF expression and that blocking the hippocampal NGF reduced the mechanical allodynia of inflamed TMJ. This suggests that the hippocampal NGF was involved in TMJ nociception and that estradiol could modulate TMJ pain through the NGF signaling pathway in the hippocampus.

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References

1. Aibel L, Martin-Zanca D, Perez P, Chao MV: Functional expression of trka receptors in hippocampal neurons. J Neurosci Res 54:424-431, 1998

2. Aloisi AM, Albonetti ME, Carli G: Formalin-induced changes in adrenocorticotropic hormone and corticosterone plasma levels and hippocampal choline acetyltransferase activity in male and female rats. Neuroscience 74: 1019-1024, 1996

3. Aloisi AM, Zimmermann M, Herdegen T: Sex-dependent effects of formalin and restraint on c-fos expression in the septum and hippocampus of the rat. Neuroscience 81: 951-958, 1997

4. Bennett DL: Neurotrophic factors: Important regulators of nociceptive function. Neuroscientist 7:13-17, 2001

5. Bereiter DA, Cioffi JL, Bereiter DF: Oestrogen receptor-immunoreactive neurons in the trigeminal sensory system of male and cycling female rats. Arch Oral Biol 50:971-979, 2005

6. Bergmann I, Reiter R, Toyka KV, Koltzenburg M: Nerve growth factor evokes hyperalgesia in mice lacking the low-affinity neurotrophin receptor p75. Neurosci Lett 255: 87-90, 1998

7. Bernabeu RO, Longo FM: The p75 neurotrophin receptor is expressed by adult mouse dentate progenitor cells and regulates neuronal and non-neuronal cell genesis. BMC Neurosci 11:136, 2010

8. Bie B, Zhang Z, Cai YQ, Zhu W, Zhang Y, Dai J, Lowenstein CJ, Weinman EJ, Pan ZZ: Nerve growth factor-regulated emergence of functional delta-opioid receptors. J Neurosci 30:5617-5628, 2010

9. Bierl MA, Jones EE, Crutcher KA, Isaacson LG: 'mature' nerve growth factor is a minor species in most peripheral tissues. Neurosci Lett 380:133-137, 2005

10. Boris-Moller F, Kamme F, Wieloch T: The effect of hypothermia on the expression of neurotrophin mrna in the hippocampus following transient cerebral ischemia in the rat. Brain Res Mol Brain Res 63:163-173, 1998

11. Brandes JL: The influence of estrogen on migraine: A systematic review. JAMA 295:1824-1830, 2006

12. Brankack J, Buzsaki G: Hippocampal responses evoked by tooth pulp and acoustic stimulation: Depth profiles and effect of behavior. Brain Res 378:303-314, 1986

13. Bruno MA, Cuello AC: Activity-dependent release of precursor nerve growth factor, conversion to mature nerve growth factor, and its degradation by a protease cascade. Proc Natl Acad Sci U S A 103:6735-6740, 2006

14. Buckwalter JA, Lappin DR: The disproportionate impact of chronic arthralgia and arthritis among women. Clin Orthop Relat Res;159-168, 2000

15. Cairns BE, Sim Y, Bereiter DA, Sessle BJ, Hu JW: Influence of sex on reflex jaw muscle activity evoked from the rat temporomandibular joint. Brain Res 957:338-344, 2002

16. Cellerino A: Expression of messenger rna coding for the nerve growth factor receptor trka in the hippocampus of the adult rat. Neuroscience 70:613-616, 1996

17. Chao MV: Neurotrophins and their receptors: A convergence point for many signalling pathways. Nat Rev Neurosci 4:299-309, 2003

18. Chuang HH, Prescott ED, Kong H, Shields S, Jordt SE, Basbaum AI, Chao MV, Julius D: Bradykinin and nerve growth factor release the capsaicin receptor from ptdins(4,5)p2-mediated inhibition. Nature 411:957-962, 2001

19. Cirillo DJ, Wallace RB, Wu L, Yood RA: Effect of hormone therapy on risk of hip and knee joint replacement in the women's health initiative. Arthritis Rheum 54:3194-3204, 2006

20. Cirulli F, Micera A, Alleva E, Aloe L: Early maternal separation increases ngf expression in the developing rat hippocampus. Pharmacol Biochem Behav 59:853-858, 1998

21. Donnerer J, Schuligoi R, Stein C: Increased content and transport of substance p and calcitonin gene-related peptide in sensory nerves innervating inflamed tissue: Evidence for a regulatory function of nerve growth factor in vivo. Neuroscience 49:693-698, 1992

22. Eriksdotter Jonhagen M, Nordberg A, Amberla K, Backman L, Ebendal T, Meyerson B, Olson L, Seiger, Shigeta M, Theodorsson E, Viitanen M, Winblad B, Wahlund LO: Intracerebroventricular infusion of nerve growth factor in three patients with alzheimer's disease. Dement Geriatr Cogn Disord 9:246-257, 1998

23. Fahnestock M, Michalski B, Xu B, Coughlin MD: The precursor pro-nerve growth factor is the predominant form of nerve growth factor in brain and is increased in alzheimer's disease. Mol Cell Neurosci 18:210-220, 2001

24. Favaro-Moreira NC, Torres-Chavez KE, Fischer L, Tambeli CH: Peripheral estradiol induces temporomandibular joint antinociception in rats by activating the nitric oxide/cyclic guanosine monophosphate signaling pathway. Neuroscience 164:724-732, 2009

25. Fischer L, Torres-Chavez KE, Clemente-Napimoga JT, Jorge D, Arsati F, de Arruda Veiga MC, Tambeli CH: The influence of sex and ovarian hormones on temporomandibular joint nociception in rats. J Pain 9:630-638, 2008

26. Fischer W, Wictorin K, Bjorklund A, Williams LR, Varon S, Gage FH: Amelioration of cholinergic neuron atrophy and spatial memory impairment in aged rats by nerve growth factor. Nature 329:65-68, 1987

27. Giovengo SL, Russell IJ, Larson AA: Increased concentrations of nerve growth factor in cerebrospinal fluid of patients with fibromyalgia. J Rheumatol 26:1564-1569, 1999

28. Grundy PL, Patel N, Harbuz MS, Lightman SL, Sharples PM: Glucocorticoids modulate the ngf mrna response in the rat hippocampus after traumatic brain injury. Brain Res 892:386-390, 2001

29. Hao J, Ebendal T, Xu X, Wiesenfeld-Hallin Z, Eriksdotter Jonhagen M: Intracerebroventricular infusion of nerve growth factor induces pain-like response in rats. Neurosci Lett 286:208-212, 2000

30. Harper RP, Kerins CA, Talwar R, Spears R, Hutchins B, Carlson DS, McIntosh JE, Bellinger LL: Meal pattern analysis in response to temporomandibular joint inflammation in the rat. J Dent Res 79:1704-1711, 2000

31. Henderson LA, Gandevia SC, Macefield VG: Gender differences in brain activity evoked by muscle and cutaneous pain: A retrospective study of single-trial fmri data. Neuroimage 39:1867-1876, 2008

32. Holmlund A, Hellsing G, Axelsson S: The temporomandibular joint: A comparison of clinical and arthroscopic findings. J Prosthet Dent 62:61-65, 1989

33. Israel HA, Diamond BE, Saed-Nejad F, Ratcliffe A: Correlation between arthroscopic diagnosis of osteoarthritis and synovitis of the human temporomandibular joint and keratan sulfate levels in the synovial fluid. J Oral Maxillofac Surg 55:210-217; discussion 217-218, 1997

34. Ji RR, Samad TA, Jin SX, Schmoll R, Woolf CJ: P38 mapk activation by ngf in primary sensory neurons after inflammation increases trpv1 levels and maintains heat hyperalgesia. Neuron 36:57-68, 2002

35. Jing YY, Wang JY, Li XL, Wang ZH, Pei L, Pan MM, Dong XP, Fan GX, Yuan YK: Nerve growth factor of red nucleus involvement in pain induced by spared nerve injury of the rat sciatic nerve. Neurochem Res 34:1612-1618, 2009

36. Kerins CA, Carlson DS, McIntosh JE, Bellinger LL: Meal pattern changes associated with temporomandibular joint inflammation/pain in rats; analgesic effects. Pharmacol Biochem Behav 75:181-189, 2003

37. Kopp S: The influence of neuropeptides, serotonin, and interleukin 1beta on temporomandibular joint pain and inflammation. J Oral Maxillofac Surg 56:189-191, 1998

38. Kou XX, Wu YW, Ding Y, Hao T, Bi RY, Gan YH, Ma X: 17beta-estradiol aggravates temporomandibular joint inflammation through the nf-kappab pathway in ovariectomized rats. Arthritis Rheum 63:1888-1897, 2011

39. Kunkler PE, Kraig RP: Hippocampal spreading depression bilaterally activates the caudal trigeminal nucleus in rodents. Hippocampus 13:835-844, 2003

40. Landi N, Manfredini D, Lombardi I, Casarosa E, Bosco M: 17-beta-estradiol and progesterone serum levels in temporomandibular disorder patients. Minerva Stomatol 53: 651-660, 2004

41. Landi N, Lombardi I, Manfredini D, Casarosa E, Biondi K, Gabbanini M, Bosco M: Sexual hormone serum levels and temporomandibular disorders. A preliminary study. Gynecol Endocrinol 20:99-103, 2005

42. Lee FS, Chao MV: Activation of trk neurotrophin receptors in the absence of neurotrophins. Proc Natl Acad Sci U S A 98:3555-3560, 2001

43. LeResche L, Mancl L, Sherman JJ, Gandara B, Dworkin SF: Changes in temporomandibular pain and other symptoms across the menstrual cycle. Pain 106:253-261, 2003

44. Lindsay RM, Harmar AJ: Nerve growth factor regulates expression of neuropeptide genes in adult sensory neurons. Nature 337:362-364, 1989

45. Martin VT, Behbehani M: Ovarian hormones and migraine headache: Understanding mechanisms and pathogenesis–part 2. Headache 46:365-386, 2006

46. Murakami K, Segami N, Fujimura K, Iizuka T: Correlation between pain and synovitis in patients with internal

derangement of the temporomandibular joint. J Oral Maxillofac Surg 49:1159-1161; discussion 1162, 1991

47. Okamoto K, Hirata H, Takeshita S, Bereiter DA: Response properties of tmj units in superficial laminae at the spinomedullary junction of female rats vary over the estrous cycle. J Neurophysiol 89:1467-1477, 2003

48. Okamoto K, Bereiter DF, Thompson R, Tashiro A, Bereiter DA: Estradiol replacement modifies c-fos expression at the spinomedullary junction evoked by temporomandibular joint stimulation in ovariectomized female rats. Neuroscience 156:729-736, 2008

49. Okifuji A, Turk DC: Sex hormones and pain in regularly menstruating women with fibromyalgia syndrome. J Pain 7:851-859, 2006

50. Ou LC, Yeh SH, Gean PW: Late expression of brain-derived neurotrophic factor in the amygdala is required for persistence of fear memory. Neurobiol Learn Mem 93: 372-382, 2010

51. Paxinos G, Watson C: The rat brain in stereotaxic coordinates. New York, NY, Academic, 1997

52. Pelegrini-da-Silva A, Oliveira MC, Parada CA, Tambeli CH: Nerve growth factor acts with the beta2-adrenoceptor to induce spontaneous nociceptive behavior during temporomandibular joint inflammatory hyperalgesia. Life Sci 83:780-785, 2008

53. Rattiner LM, Davis M, French CT, Ressler KJ: Brain-derived neurotrophic factor and tyrosine kinase receptor b involvement in amygdala-dependent fear conditioning. J Neurosci 24:4796-4806, 2004

54. Raychaudhuri SP, Sanyal M, Weltman H, Kundu-Raychaudhuri S: K252a, a high-affinity nerve growth factor receptor blocker, improves psoriasis: An in vivo study using the severe combined immunodeficient mouse-human skin model. J Invest Dermatol 122:812-819, 2004

55. Ren K: An improved method for assessing mechanical allodynia in the rat. Physiol Behav 67:711-716, 1999

56. Saporito MS, Carswell S: High levels of synthesis and local effects of nerve growth factor in the septal region of the adult rat brain. J Neurosci 15:2280-2286, 1995

57. Sarchielli P, Alberti A, Floridi A, Gallai V: Levels of nerve growth factor in cerebrospinal fluid of chronic daily headache patients. Neurology 57:132-134, 2001

58. Scharfman HE, Mercurio TC, Goodman JH, Wilson MA, MacLusky NJ: Hippocampal excitability increases during the estrous cycle in the rat: A potential role for brain-derived neurotrophic factor. J Neurosci 23:11641-11652, 2003

59. Son JH, Winzer-Serhan UH: Chronic neonatal nicotine exposure increases mrna expression of neurotrophic factors in the postnatal rat hippocampus. Brain Res 1278: 1-14, 2009

60. Sowers MR, McConnell D, Jannausch M, Buyuktur AG, Hochberg M, Jamadar DA: Estradiol and its metabolites and their association with knee osteoarthritis. Arthritis Rheum 54:2481-2487, 2006

61. 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 19:34-40, 2005

62. Svensson P, Wang MW, Dong XD, Kumar U, Cairns BE: Human nerve growth factor sensitizes masseter muscle nociceptors in female rats. Pain 148:473-480, 2010

63. Tapley P, Lamballe F, Barbacid M: K252a is a selective inhibitor of the tyrosine protein kinase activity of the trk family of oncogenes and neurotrophin receptors. Oncogene 7: 371-381, 1992

64. Tashiro A, Okamoto K, Bereiter DA: Chronic inflammation and estradiol interact through mapk activation to affect tmj nociceptive processing by trigeminal caudalis neurons. Neuroscience 164:1813-1820, 2009

65. Tian YF, Zhang PB, Xiao XL, Zhang JS, Zhao JJ, Kang QY, Chen XL, Qiu F, Liu Y: The quantification of adamts expression in an animal model of cerebral ischemia using real-time pcr. Acta Anaesthesiol Scand 51:158-164, 2007

66. Wang YJ, Wang X, Lu JJ, Li QX, Gao CY, Liu XH, Sun Y, Yang M, Lim Y, Evin G, Zhong JH, Masters C, Zhou XF: P75ntr regulates abeta deposition by increasing abeta production but inhibiting abeta aggregation with its extracellular domain. J Neurosci 31:2292-2304, 2011

67. Warren MP, Fried JL: Temporomandibular disorders and hormones in women. Cells Tissues Organs 169:187-192, 2001

68. Weskamp G, Otten U: An enzyme-linked immunoassay for nerve growth factor (ngf): A tool for studying regulatory mechanisms involved in ngf production in brain and in peripheral tissues. J Neurochem 48:1779-1786, 1987

69. Woolf NJ, Milov AM, Schweitzer ES, Roghani A: Elevation of nerve growth factor and antisense knockdown of trka receptor during contextual memory consolidation. J Neurosci 21:1047-1055, 2001

70. Wu YW, Bi YP, Kou XX, Xu W, Ma LQ, Wang KW, Gan YH, Ma XC: 17-beta-estradiol enhanced allodynia of inflammatory temporomandibular joint through upregulation of hippocampal trpv1 in ovariectomized rats. J Neurosci 30: 8710-8719, 2010

71. Xue Q, Jong B, Chen T, Schumacher MA: Transcription of rat trpv1 utilizes a dual promoter system that is positively regulated by nerve growth factor. J Neurochem 101: 212-222, 2007

72. Yan J, Welsh AM, Bora SH, Snyder EY, Koliatsos VE: Differentiation and tropic/trophic effects of exogenous neural precursors in the adult spinal cord. J Comp Neurol 480: 101-114, 2004

73. Zhang X, Huang J, McNaughton PA: Ngf rapidly increases membrane expression of trpv1 heat-gated ion channels. Embo J 24:4211-4223, 2005