Central Role of Protein Kinase A in Promoting Trigeminal Nociception in an In Vivo Model of Temporomandibular Disorders

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Aims: To investigate cellular changes in the spinal trigeminal nucleus (STN) and trigeminal ganglion (TG) associated with trigeminal nociception mediated by inflammation in the temporomandibular joint (TMJ). **Methods:** Male Sprague-Dawley rats (n = 86) were utilized to investigate cellular and behavioral responses to prolonged TMJ inflammation caused by bilateral injection of Complete Freund's Adjuvant (CFA) in the TMJ capsules. To investigate the cellular effects of protein kinase A (PKA) in the STN, rats were injected intrathecally with the selective PKA inhibitor KT5720 prior to injection of CFA into both TMJ capsules. Levels of calcitonin gene-related peptide (CGRP), active PKA, and ionized calcium-binding adapter molecule 1 (Iba1) in the STN and expression of phosphorylated extracellular regulated kinases (p-ERK) in the TG were determined with immunohistochemistry (n ≥ 3 experiments per test condition). Nocifensive head withdrawal responses to mechanical stimulation of the cutaneous tissue over the TMJ were monitored following CFA injection in the absence or presence of KT5720 (n = 7). Statistical analysis was performed using parametric analysis of variance (ANOVA) tests. **Results:** Intrathecal injection of KT5720 significantly inhibited the stimulatory effect of CFA on levels of CGRP, PKA, and Iba1 in the STN. In addition, administration of KT5720 decreased the average number of CFA-induced nocifensive withdrawal responses to mechanical stimulation and the CFA-mediated increase in p-ERK expression in the ganglion. **Conclusion:** These findings provide evidence that elevated PKA activity in the STN promotes cellular events temporally associated with trigeminal nociception caused by prolonged TMJ inflammation. *J Oral Facial Pain Headache 2017;31:264–274. doi: 10.11607/ofph.1803*

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Temporomandibular disorders (TMD), which affect between 5% and 12% of the adult population, are characterized by pain or tenderness in the temporomandibular joint (TMJ) and/or jaw muscles during or after mastication, as well as jaw clicking, limited jaw movement, tinnitus, and headache.[1–4] TMD are at least twice as prevalent in women as in men, with the incidence of pain highest during adolescence.[5] Individuals with TMD often exhibit increased sensitivity to other experimentally induced pains.[6,7] The pathologic pain associated with TMD involves activation of trigeminal ganglion (TG) nerves, which provide sensory innervation of the head and face and relay nociceptive signals to the spinal trigeminal nucleus (STN).[8–10] Peripheral and central sensitization of trigeminal nociceptive neurons are associated with the pathology of prevalent and debilitating orofacial pain conditions, including TMD.[10] Following peripheral activation of trigeminal nerves in response to tissue injury, calcitonin gene-related peptide (CGRP) and other inflammatory mediators can induce cellular changes in second-order neurons and glial cells involved in the initiation and maintenance of central sensitization and persistent pain.[11,12] Elevated CGRP levels in the spinal cord are implicated in the development of central sensitization

through the mediation of changes in the expressions of ion channels, receptors, and inflammatory genes in second-order neurons and glial cells, including astrocytes and microglia. Activation of astrocytes and microglia, which results in a prolonged inflammatory response, facilitates sustained central sensitization and promotes a pathologic pain state.[10,13–15]

CGRP is involved in the initiation and maintenance of central sensitization via activation of CGRP receptors that are localized on second-order neurons and glial cells within the spinal cord.[16–18] Based on prior studies, activation of CGRP receptors in neurons and glial cells would likely lead to an increase in intracellular levels of the secondary messenger cyclic adenosine monophosphate (cAMP), which binds to and stimulates activation of protein kinase A (PKA).[19–22] The signaling protein PKA induces expression of proinflammatory genes that are involved in sustaining a sensitized state of second-order neurons, including cytokines.[23] Elevated PKA activity in the cytosol is correlated with sensitization and activation of nociceptive neurons and glial cells via modulation of receptor expression and ion channel activity.[11,24] CGRP is also known to cause activation of the mitogen-activated protein (MAP) kinases, including p38, c-Jun kinase (JNK), and extracellular regulated kinase (ERK), in trigeminal neurons and glia[25,26] that facilitate an inflammatory response in the TG associated with sensitization of trigeminal neurons.[27] Similar to PKA, increased expression of these signaling proteins leads to a prolonged state of sensitization via modulation of ion channels, receptors, and transcription factors. Given the importance of the CGRP/PKA pathway in promoting increased neuron-glia communication, a goal of this study was to better understand the role of these cellular events implicated in peripheral and central sensitization of trigeminal nociceptive neurons in TMD pathology.

Several animal models of orofacial pain have been developed that mimic certain aspects of TMD to study the mechanisms involved in promoting prolonged trigeminal sensitization and inflammation by injection of inflammatory agents into the joint capsule. Capsaicin is often used to induce a transient state of sensitization characterized by hyperalgesia that lasts about 24 hours and fully resolves within 2 to 3 days. A more chronic sensitization state can be established using Complete Freund's Adjuvant (CFA), which is an emulsion of heat-killed *Mycobacterium tuberculosis*. Findings from previous studies have shown that CFA can cause sustained induction and maintenance of peripheral and central sensitization for more than 14 days postinjection.[28–30] The aim of this study was to investigate cellular changes in the STN and TG associated with trigeminal nociception mediated by inflammation in the TMJ.

Materials and Methods

Animals

A total of 86 adult male Sprague-Dawley rats (350–500 g) were obtained from Charles River Laboratories Inc or purchased from Missouri State University (internal breeding colonies). Upon arrival, animals were acclimated to the environment for a minimum of 1 week prior to use. Animals were housed individually in clean, standard plastic rat cages (VWR) with unrestricted access to both food and water in a room with 12-hour light/dark cycles. 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, US 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.

Reagents

CFA (Sigma-Aldrich) was prepared as a 1:1 emulsion in 0.9% saline solution immediately prior to use. The selective signaling inhibitor of PKA, KT5720 (Tocris), was prepared at a stock concentration of 1 mM in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and frozen in 5-µl aliquots at −20°C. On the day of the experiment, a fresh 500-nM solution of KT5720 was prepared via dilution in 0.9% saline.

Immunohistochemistry

To investigate changes in protein expression, immunohistochemical analysis of the brainstem, spinal cord, and TG tissues was performed as described previously.[28,31] Animals were anesthetized by inhalation of 5% isoflurane. To promote inflammation in the TMJ, 50 mL of CFA (1:1 CFA/0.9% saline emulsion) was injected into each capsule by using a 26.5-gauge needle (Becton Dickinson) and a 50-µL Hamilton syringe (Hamilton Company). Some animals were injected intrathecally between the occipital bone and the first cervical vertebra (C1) with 20 µl of KT5720 (500 nM) or a 0.9% saline solution immediately prior to bilateral TMJ injections. The naïve controls received no treatment. Immunohistochemical data were obtained from 13 animals receiving only CFA, 9 animals receiving CFA + KT5720, 10 animals receiving isotonic saline, 9 animals receiving only KT5720, and 15 naïve control animals.

The brainstem and upper spinal cord (6 mm posterior to the obex) were removed at 2 hours, 7 days, or 21 days after injection. Right and left TG tissues were also acquired through cranial dissection 2 hours post–CFA injection. Immediately after removal, tissues were incubated in 4% paraformaldehyde at 4°C overnight and then washed in 1× phosphate-buffered

Table 1 Summary of Antibodies and Conditions Used for Immunohistochemistry

Protein	Dilution	Incubation time	Incubation temperature	Company	Location
CGRP	1:1,000	3 h	20–22°C	Sigma-Aldrich, Inc	St. Louis, Missouri
Iba-1	1:400	3 h	20–22°C	Wako Chemicals USA, Inc	Richmond, Virginia
NeuN	1:1,000	3 h	20–22°C	EMD Millipore, Corp	Temecula, California
p-ERK	1:500	Overnight	4°C	Bioworld Technology, Inc	St. Louis Park, Minnesota
Active PKA	1:500	3 h	20–22°C	Abcam, Inc	Cambridge, Massachusetts
Alexa Fluor 488	1:200	1 h	20–22°C	Jackson ImmunoResearch, Inc	West Grove, Pennsylvania
Alexa Fluor 568	1:200	1 h	20–22°C	Life Technologies	Grand Island, New York
Alexa Fluor 647	1:200	1 h	20–22°C	Life Technologies	Grand Island, New York

saline (PBS) before being placed in 12.5% sucrose for 1 hour at 4°C and then in 25% sucrose at 4°C overnight. At this point, they were removed from sucrose and stored at –20°C. Before sectioning, tissues were embedded in Optimal Cutting Temperature (OCT) compound (Sakura Finetek). Transverse sections 14 µm thick were taken between 4 mm and 5 mm caudal to the obex of the STN with a cryostat set to –24°C. Transverse sections 14 µm thick were also taken from the middle of the TG tissue, which contains all three branches of the ganglion. Sections were placed on Superfrost Plus microscope slides (Fisher Scientific) with the caudal side facing down and stored at –20°C.

Slides with sectioned tissues were rehydrated by incubation in PBS for 5 minutes, then blocked and permeabilized in a solution of 0.1% Triton X-100 in 5% donkey serum (Jackson ImmunoResearch Laboratories) for 20 minutes at room temperature. Primary antibodies were prepared according to the manufacturer's recommended dilutions (Table 1) in 5% donkey serum. Slides were thoroughly rinsed with PBS and the primary antibodies were prepared and incubated for either 3 hours at room temperature or overnight at 4°C in a humidified chamber. Next, slides were incubated for 1 hour at room temperature with Donkey Alexa Fluor conjugated secondary antibodies diluted in PBS. Vectashield medium (H-1200, Vector Laboratories Inc) containing 4',6-diamidino-2-phenylindole (DAPI) was used to mount the tissue sections and to visualize cell nuclei with fluorescent microscopy. A Zeiss Axiocam mRm camera (Carl Zeiss Microscopy) mounted on a Zeiss Imager Z2 fluorescent microscope equipped with an ApoTome was used to collect 100× images of the outer lamina of the STN or the V3 branch of the TG. Image acquisition was performed using Zeiss Zen 2012 software. No specific immunostaining pattern was observed when the protocol was conducted in the absence of primary antibodies, providing evidence of the specificity of the fluorescently conjugated antibodies.

Nocifensive Responses to Mechanical Stimulation

Mechanical nociception was evaluated using calibrated von Frey filaments (Ugo Basile). All behavioral procedures were conducted between the hours of 7 am and 11 am. Behavioral assessments were performed as described in previously published studies using the Durham Animal Holder (Ugo Basile).[28,31,32] The animals were gently guided into this device and secured using a plastic blockade inserted behind the hind paws. To minimize false responses during von Frey filament testing, a pipette tip was used to touch the animal's head and face to acclimate the rats to having the cutaneous tissue over the TMJ capsule touched with a filament. This was done for 3 consecutive days prior to testing done with von Frey filaments.

Following the acclimation period, baseline response levels to a series of calibrated von Frey filaments (North Coast Medical, Inc; 26, 60, 100, and 180 g) were determined 24 hours prior to TMJ procedures through application to the cutaneous tissue over the TMJ capsule. An experimenter blinded to the experimental conditions applied the filaments bilaterally in order of increasing pressure with five applications per filament. A positive response was recorded and verified by another experimenter when the animal visibly flinched away from the filament prior to it bending. The day after establishing baseline mechanical sensitivity, the animals were anesthetized and treated as described for the immunohistochemistry studies. The nocifensive head withdrawal responses to mechanical stimulation were determined at 2 hours, 1 day, 5 days, 7 days, 14 days, and 21 days following treatment. Behavioral data were obtained from seven CFA-only animals, seven CFA + KT5720 animals, six saline animals, four KT5720-only animals, and six naïve control animals.

Data and Statistical Analyses

Statistical analyses were performed as described in previous published studies.[28,31,33] Immunohistochemical analyses were performed and

confirmed by at least two experimenters blinded to experimental conditions. For immunohistochemical analysis of the brainstem and spinal cord (n ≥ 3 independent experiments per test condition), relative levels of the proteins of interest were analyzed using NIH image J software. Fluorescent intensity was measured in 10 nonoverlapping rectangular regions in laminas I–III of the STN. To normalize intensity measurements within each image, background intensity values were obtained from 10 nonoverlapping regions in the acellular area of the outer lamina as determined by DAPI, and average values were subtracted from the region of interest staining intensity values. All data are presented as mean fold change from the average naïve value ± standard error of the mean (SEM). Analysis was performed using one-way analysis of variance (ANOVA) with Games-Howell post hoc test due to unequal variances or Tukey post hoc test in the case of equal variances, as determined by Levene's test. To quantify p-ERK expression in the TG, the number of neuronal cells exhibiting nuclear localization of p-ERK was divided by the total number of visible neuronal nuclei as identified by DAPI and NeuN staining. Results are reported as the average percent ± SEM of neurons with p-ERK nuclear staining. Statistical significance was set at $P < .05$.

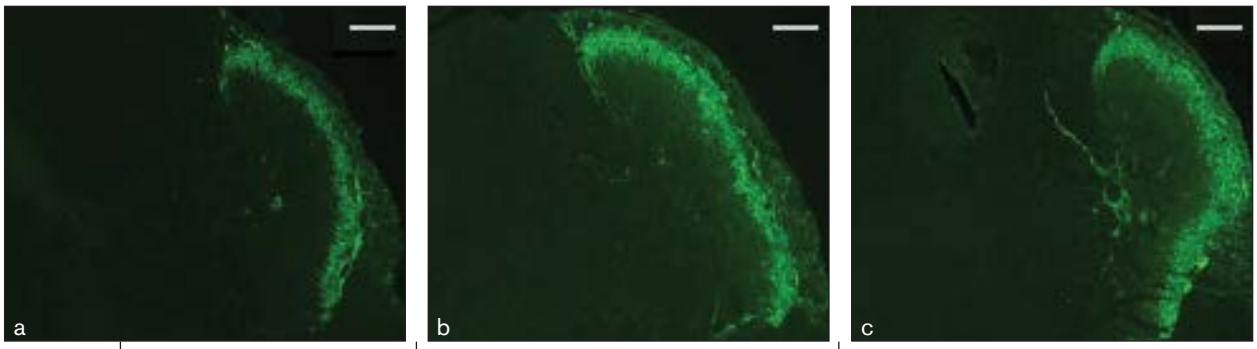
For the mechanical sensitivity studies, the data are reported as the mean number of withdrawal responses ± SEM to 100 g of force for each test condition and time point. Subsequent analysis was then performed on data, with n ≥ 6 for each experimental condition, using a mixed design repeated measures ANOVA to test for general statistical significance, followed by a paired samples *t* test to find changes within subjects from baseline and an independent samples *t* test to test for differences between groups. Statistical significance was set at $P < .05$.

Results

The inhibitory molecule KT5720 was used to determine whether the stimulatory effect of CFA-mediated inflammation in the TMJ involved upregulation of CGRP and activation of the PKA pathway. Tissues from naïve, unstimulated animals exhibited low levels of CGRP in the outer lamina of the STN (Fig 1). At 2 hours after injections, the saline control group showed no detectable differences in staining intensity when compared to the levels in the naïve control group (0.96 ± 0.06 fold; $P = .958$). The levels in the saline animals were significantly different than those in the naïve animals at day 7 (1.27 ± 0.13 fold; $P < .001$), but were again similar at day 21 (1.14 ± 0.08 fold; $P = .447$). Compared to the saline and naïve control groups, intrathecal delivery of the selective PKA inhibitor KT5720 exhibited no detectable differences in CGRP levels at 2 hours or 7 days posttreatment, but levels were significantly lower at 21 days compared to both saline (0.65 ± 0.014 fold; $P < .001$) and naïve (0.74 ± 0.05 fold, $P < .001$) control animals. The CFA-only animals exhibited elevated CGRP levels compared to saline control animals at 2 hours (1.70 ± .05 fold, $P < .001$), 7 days (1.29 ± .04 fold; $P < .001$), and 21 days (1.47 ± .04 fold, $P < .001$) after injections, as well as compared to the naïve animals ($P < .001$) (Fig 1). CFA + KT5720 injection was associated with lower levels of CGRP immunostaining compared to the CFA-only animals at 2 hours (1.45 ± 0.05 fold, $P < .001$). At day 7, the CFA + KT5720 animals exhibited CGRP levels significantly lower than in CFA-only animals (0.68 ± 0.04 fold; $P < .001$) and similar to those in naïve control animals ($P = .501$) (Fig 1). At 21 days after treatment, CFA + KT5720 animals continued to express levels of CGRP similar to those observed in naïve control animals ($P = .053$), but less than those in CFA-only animals (0.50 ± 0.02 fold) ($P < .001$) (Fig 1).

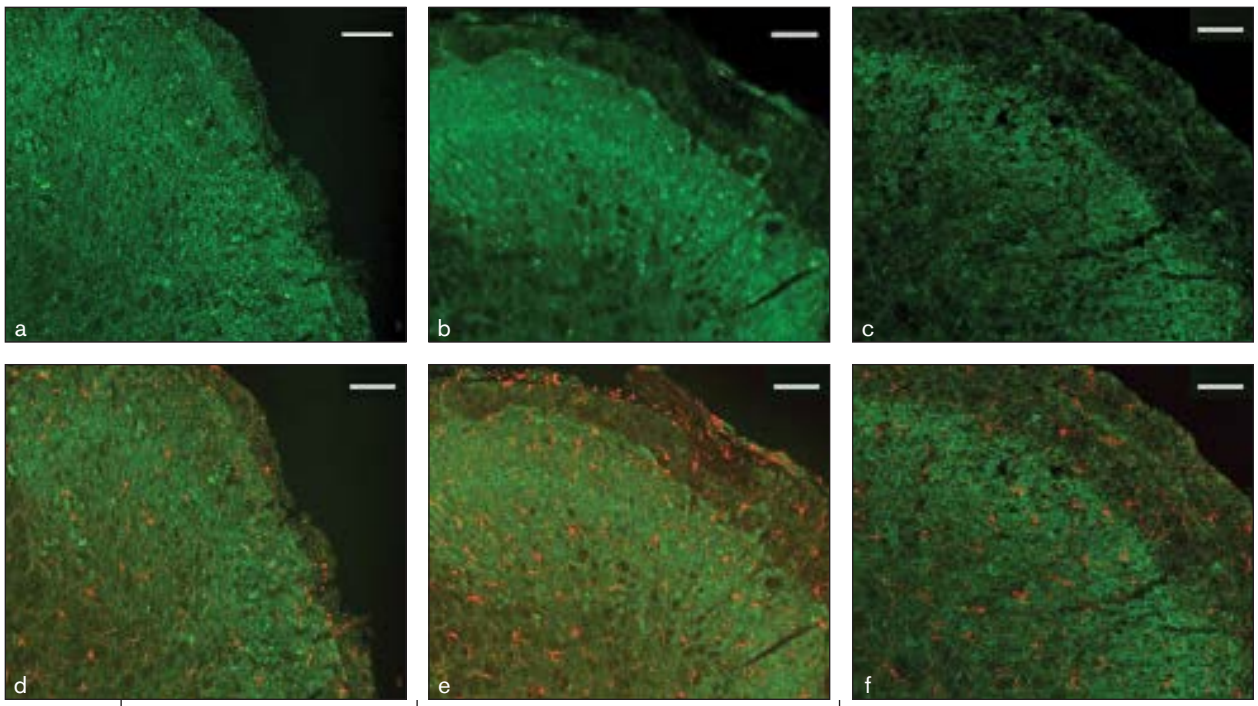
Levels of the active form of PKA were evaluated to determine the amount of intracellular signaling occurrences that promote neuron-glia interactions and maintain a hyperexcitable state in nociceptive neurons. In naïve control animals, low levels of PKA immunostaining were detected within the STN (Fig 2). The saline control animals showed no significant difference from the naïve control animals at 2 hours (1.15 ± 0.08 fold; $P = .331$), 7 days (1.02 ± 0.10 fold; $P = .999$), or 21 days (0.98 ± 0.09 fold; $P = .981$) postinjection. The KT5720-only animals did not exhibit detectable differences from naïve PKA levels at 2 hours (1.17 ± 0.04 fold; $P = .068$), 7 days (0.82 ± 0.09 fold; $P = .075$), or 21 days postinjection (1.05 ± 0.09 fold; $P = .847$). CFA-only animals showed an increase in PKA immunostaining intensity at 2 hours postinjection compared to saline controls (3.11 ± .015 fold; $P < .001$), which persisted through day 7 (2.79 ± 0.13 fold, $P < .001$) and day 21 (2.63 ± 0.14 fold, $P < .001$), as well as with the naïve controls ($P < .001$) (Fig 2).

Based on co-staining, PKA was detectable in Iba1-positive microglial cells (Fig 2) as well as in NeuN-positive neurons in CFA-only animals (data not shown). At 2 hours, the CFA + KT5720 animals showed PKA levels that were significantly lower than those in CFA-only animals (0.29 ± 0.02 fold; $P < .001$) and similar to those in naïve controls ($P = .435$) (Fig 2). This changed on day 7, when PKA levels were significantly increased compared to levels in saline ($P = .001$) and naïve ($P < .001$) control animals, although they remained significantly lower than those in CFA-only animals (2.79 ± 0.13; $P < .001$).



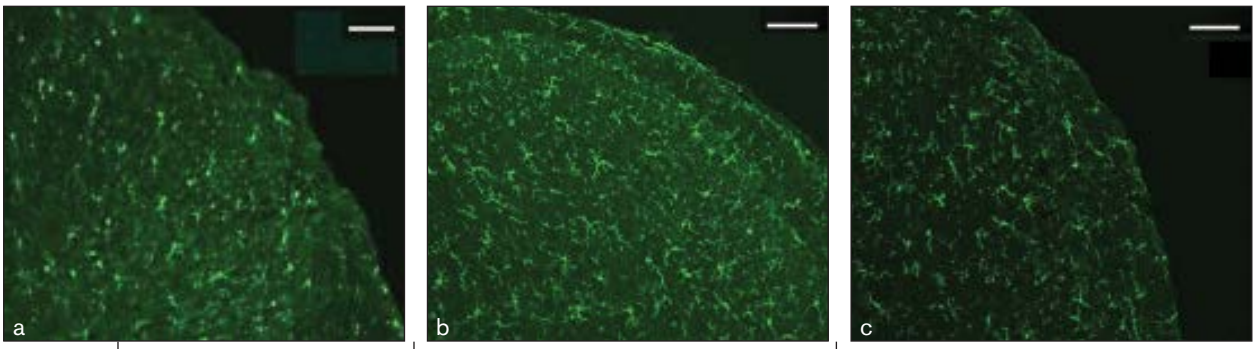
Time point	Naïve	CFA	CFA + KT5720
2 h	1.00 ± 0.04	1.63 + 0.07[a]	1.45 ± 0.05[a]
Day 7	1.00 ± 0.05	1.64 + 0.06[a]	1.12 ± 0.07[b]
Day 21	1.00 ± 0.05	1.67 + 0.07[b]	0.83 ± 0.06[b]

Fig 1 CFA stimulation of CGRP expression in the STN is repressed by inhibition of PKA activity. Representative images of sections from the STN obtained at the 2-hour time point from naïve animals **(a)**, animals receiving CFA **(b)**, and animals receiving CFA and intrathecal administration of KT5720 **(c)** are shown, with the reported average change in relative staining intensity for CGRP at 2 hours, 7 days, and 21 days after injections. Data are reported as mean fold change ± SEM from levels in naïve control animals, whose mean was made equal to 1. [a]$P < .05$ when compared to levels in naïve control animals. [b]$P < .05$ when compared to levels in CFA-only animals. Scale bar = 200 μm.



Time point	Naïve	CFA	CFA + KT5720
2 h	1.00 ± 0.07	3.58 + 0.12[a]	1.01 ± 0.08[b]
Day 7	1.00 ± 0.07	2.85 + 0.16[a]	1.45 ± 0.10[a,b]
Day 21	1.00 ± 0.07	2.58 + 0.15[a]	1.00 ± 0.09[b]

Fig 2 Inhibition of PKA signaling in the STN repressed CFA-mediated increase in PKA immunoreactive levels. Representative images of sections from the STN obtained at the 2-hour time point from naïve animals **(a)**, animals receiving CFA **(b)**, or animals receiving CFA injection and intrathecal administration of KT5720 **(c)** and stained for the active form of PKA (green) are shown in the top panels. The same images co-stained for the expression of Iba1 (red) are presented, respectively, in the lower panels **(d–f)**. The average change in relative staining intensity of PKA from levels in naïve control animals at 2 hours, 7 days, and 21 days after injections is reported. Data are reported as mean fold change ± SEM from levels in naïve control animals, whose mean was made equal to 1. [a]$P < .05$ when compared to levels in naïve control animals. [b]$P < .05$ when compared to levels in CFA-only animals. Scale bar = 100 μm.



Time point	Naïve	CFA	CFA + KT5720
2 h	1.00 ± 0.08	1.50 + 0.09[a]	0.69 ± 0.10[a,b]
Day 7	1.00 ± 0.06	2.91 + 0.20[a]	1.11 ± 0.12[b]
Day 21	1.00 ± 0.05	1.82 + 0.14[a]	0.69 ± 0.09[a,b]

Fig 3 CFA-mediated increase in Iba1 in microglia is inhibited by PKA inhibitor KT5720. Representative images of sections of the STN obtained at the 2-hour time point from naïve animals **(a)**, animals receiving CFA **(b)**, or animals receiving CFA injection and intrathecal administration of KT5720 **(c)** are shown. The average change in relative staining intensity of Iba1 from levels in naïve control animals at 2 hours, 7 days, and 21 days after injections is reported. Data are reported as a mean fold change ± SEM from levels in naïve animals, whose mean was made equal to 1. [a]$P < .05$ when compared to levels in naïve control animals. [b]$P < .05$ when compared to levels in CFA-only animals. Scale bar = 100 μm.

The CFA + KT5720 animals again displayed PKA levels that were similar to levels in saline (1.02 ± 0.02 fold; $P = 1.00$) and naïve ($P = 1.00$) control animals at 21 days postinjection, and these were significantly lower than the elevated levels mediated by CFA (0.39 ± 0.01; $P < .001$) (Fig 2).

Saline control animals exhibited low levels of Iba1 staining in the STN that were not significantly different from naive levels 2 hours (1.15 ± 0.13 fold, $P = .778$), 7 days (1.07 ± 0.14 fold, $P = 1.00$), and 21 days (1.05 ± 0.09 fold, $P = .99$) postinjection. Animals treated with KT5720 alone displayed no detectable differences from naïve control animals 2 hours after injection (1.03 ± 0.11 fold, $P = .993$), but on day 7, displayed significantly less Iba1 staining compared to naïve and saline control animals (0.72 ± 0.08 fold, $P = .001$, and 0.67 ± 0.08 fold, $P = .002$, respectively). However, this decrease in Iba1 expression was no longer significant at 21 days postinjection (0.76 ± 0.10 fold, $P = .10$). CFA-only animals showed an increase in Iba1 immunostaining intensity compared to both naïve and saline control animals at 2 hours (1.30 ± 0.04); these levels remained significantly increased in CFA-only animals on days 7 (2.71 ± 0.18) and 21 (1.73 ± 0.12; $P = .017$ for both). The CFA + KT5720 animals had relative intensity levels significantly lower than those in saline (0.6 ± 0.06 fold; $P = .003$), CFA-only (0.46 ± 0.04 fold; $P < .001$), and naïve ($P = .037$) animals at 2 hours postinjection (Fig 3). However, at 7 days, Iba1 levels were increased slightly in CFA + KT5720 animals to levels similar to those of naïve and saline controls ($P = .894$ for both). On day 21, Iba1 expression was significantly reduced to levels below naïve controls ($P = .025$) (Fig 3).

Behavioral testing was performed to determine whether cellular changes in the STN mediated by inhibition of PKA signaling would be associated with an analogous reduction in nocifensive behavior. Responses to the 100-g von Frey filament were used to assess nocifensive behavior, since animals rarely responded to this force at baseline readings but consistently responded to the subsequent 180-g filament. The average numbers of nocifensive head withdrawals by animals for both the left and right sides to the 100-g filament at 2 hours, 1 day, 5 days, 7 days, 14 days, and 21 days in naïve, isotonic saline, CFA-only, and CFA + KT5720 animals are shown in Fig 4. All naïve animals withdrew from the 100-g filament on average 0.5 ± 0.1 times at baseline, 1.1 ± 0.4 at 2 hours, 1.2 ± 0.8 on day 1, 0.7 ± 0.2 on day 5, 1.0 ± 0.3 on day 7, 1.1 ± 0.2 on day 14, and 1.0 ± 0.4 on day 21. Animals injected intrathecally with saline withdrew on average 0.6 ± 0.2 times at baseline, 1.3 ± 0.3 at 2 hours postinjection, 0.9 ± 0.2 on day 1, 0.8 ± 0.3 on day 5, 0.9 ± 0.3 on day 7, 1.1 ± 0.3 on day 14, and 0.8 ± 0.3 times on day 21. The KT5720-only animals withdrew on average 0.5 ± 0.4 times at baseline, 1.5 ± 0.0 at 2 hours postinjection, 0.9 ± 0.4 at day 1, 0.5 ± 0.2 at day 5, 0.5 ± 0.4 at day 7, 0.4 ± 0.2 at day 14, and 1.0 ± 0.4 times at day 21 (data not shown). No animals from any of the control conditions at any time point exhibited nocifensive responses that

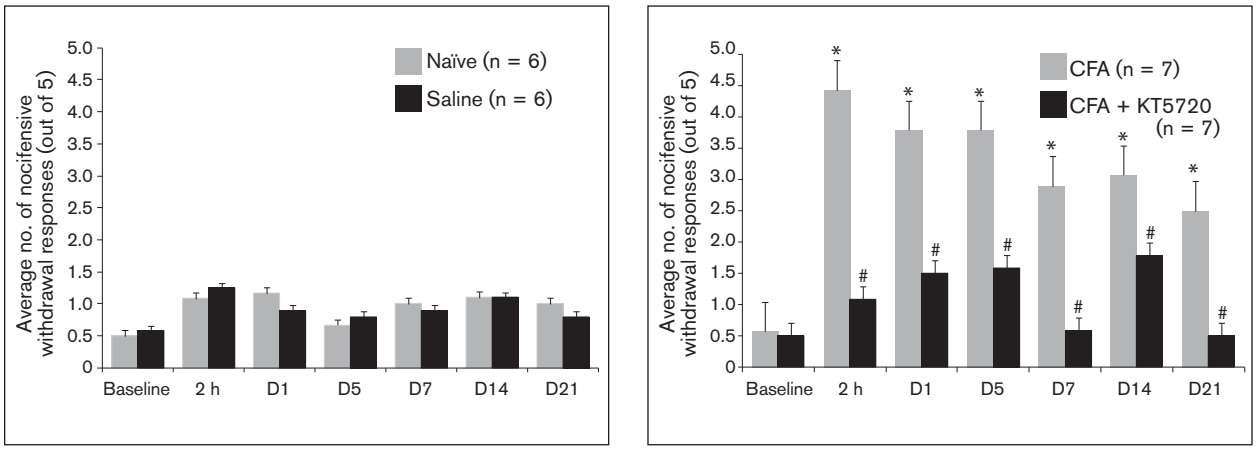


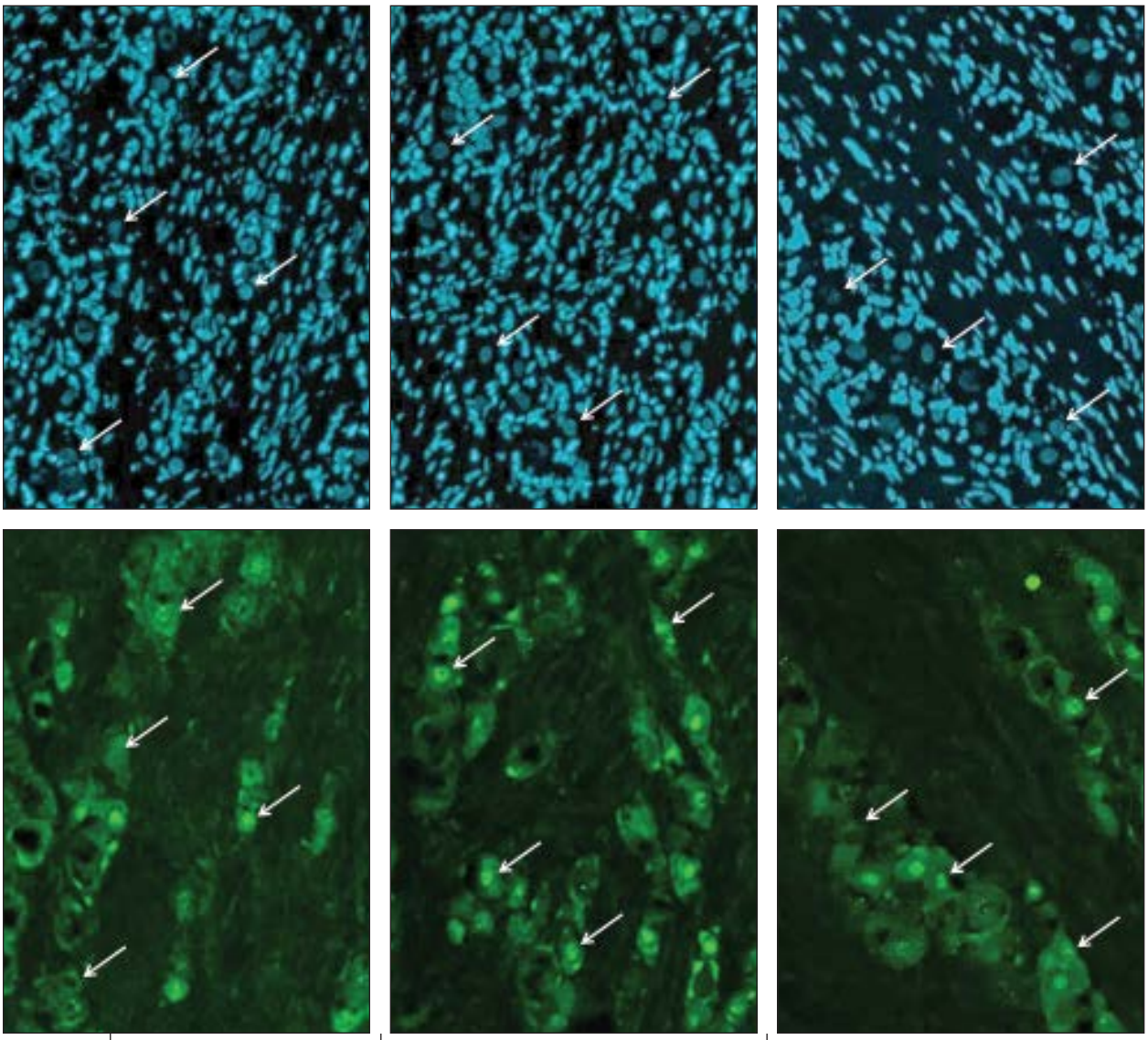
Fig 4 Inhibition of PKA signaling is sufficient to repress nocifensive head withdrawal responses to mechanical stimulation after CFA injection. Average nocifensive withdrawal responses ± SEM to 100-g force applied to the cutaneous area over the TMJ at 2 hours and 1, 5, 7, 14, and 21 days after intrathecal injection of sterile saline *(left)* or CFA and intrathecal KT5720 *(right)* when compared to levels in naïve control animals. [a]$P < .05$ when compared to baseline levels. [b]$P < .05$ when compared to CFA-only values.

reached statistical significance from baseline values. In contrast, CFA injection into the TMJ capsule resulted in a significant increase in the average number of nocifensive responses compared to naïve and saline control animals, respectively, at 2 hours ($P < .001$ for both), day 1 ($P = .007$ and $P < .001$), day 5 ($P < .001$ and $P < .001$), day 7 ($P = .001$ and $P = .002$), and day 14 ($P = .013$ and $P = .024$). CFA-only animals withdrew on average 0.6 ± 0.1 times at baseline, 4.4 ± 0.4 at 2 hours postinjection, 3.8 ± 0.4 on day 1, 3.8 ± 0.4 on day 5, 2.9 ± 0.3 on day 7, 3.1 ± 0.7 on day 14, and 2.5 ± 0.7 on day 21. In addition, CFA-only animals displayed a significant increase in the average number of withdrawal responses compared to their own baseline levels at every time point postinjection: 2 hours ($P < .001$); day 1 ($P < .001$); day 5 ($P < .001$); day 7 ($P = .001$); day 14 ($P = .003$); and day 21 ($P = .034$). CFA + KT5720 animals exhibited a significant inhibition of nocifensive behavior across all time points postinjection. CFA + KT5720 animals responded to the 100-g filament on average 0.5 ± 0.1 times at baseline, 1.1 ± 0.3 at 2 hours postinjection, 1.5 ± 0.6 on day 1, 1.6 ± 0.6 on day 5, 0.6 ± 0.3 on day 7, 1.8 ± 0.6 on day 14, and 0.5 ± 0.4 on day 21. At every time point, the average number of withdrawal responses by the CFA + KT5720 animals were similar to values reported for the naïve and saline control animals.

The significant reduction in nocifensive responses mediated by the PKA inhibitor prompted investigation of cellular changes in the peripheral TG neurons. Activation of peripheral nociceptors was evaluated with immunohistochemical analysis of p-ERK expression in the nuclei of DAPI-positive neuronal cell bodies in the ganglion. In tissue from naïve animals, the average amount of neurons exhibiting elevated nuclear levels of p-ERK after 2 hours was 19.4% ± 2.7%; this trend remained consistent at both subsequent time points (Fig 5). Levels were similar in saline and naïve control animals at 2 hours, day 7, and day 21. Tissues from CFA + KT5720 animals also expressed low p-ERK levels at 2 hours (11.7% ± 1.26%, $P = .574$), day 7 (20.9% ± 1.61%, $P = .763$), and day 21 (20.3% ± 4.8%, $P = .990$). In contrast, CFA-only animals displayed significantly elevated expression compared to naïve and saline control animals at 2 hours ($P < .001$ for both), day 7 ($P = .001$ and $P = .002$, respectively), and day 21 ($P = .002$ and $P = .001$, respectively). p-ERK levels in CFA + KT5720 animals were significantly repressed compared to CFA-only animals at 2 hours ($P < .001$), day 7 ($P = .002$), and day 21 ($P = .006$). Nuclear p-ERK levels in ganglia from animals receiving CFA + KT5720 were not significantly different when compared to naïve at any time point ($P = .989$ at 2 hours, $P = .997$ on day 7, $P = .988$ on day 21).

Discussion

The main goal of this study was to investigate the role of PKA in promoting cellular changes in trigeminal STN neurons associated with nociception in an established in vivo model of TMD. The inflammatory mediator CFA was injected into both TMJ capsules to mimic prolonged TMD pathology mediated by sustained inflammation promoting a persistent state of trigeminal sensitization.[28,34–38] The emulsion



Time point	Naïve	CFA	CFA + KT5720
2 h	19.4 ± 2.7	52.4 + 3.9[a]	21.7 ± 3.2[b]
Day 7	18.2 ± 2.1	51.1 + 8.3[a]	20.1 ± 4.0[b]
Day 21	22.4 ± 2.0	47.1 + 5.2[a]	24.6 ± 5.6[b]

Fig 5 Increased nuclear p-ERK expression in TG neurons in response to CFA was inhibited by KT5720. Representative images of sections from the V3 region of the TG obtained from naïve *(left)*, CFA-only *(center)*, and CFA + KT5720 *(right)* animals at 2 hours after injections are shown. All cell nuclei are identified by the nuclear dye DAPI *(top panels)*. The same images were co-stained for p-ERK *(bottom panels)*. Arrows indicate neuronal cell body nuclei identified by DAPI. The average percent ± SEM of p-ERK positive neuronal nuclei, as identified by DAPI staining, for each condition is reported. [a]$P < .05$ when compared to levels in naïve control animals. [b]$P < .05$ when compared to levels in saline control animals.

of heat-killed *M tuberculosis* elicits a robust innate immune response characterized by the recruitment of proinflammatory mediators, including cytokines, chemokines, and CGRP, that promote prolonged edema and pain localized to the TMJ.[37,39] In addition to a local inflammatory response, TMD is characterized by the development of peripheral and central sensitization of trigeminal nociceptive neurons.[10] The neuropeptide CGRP is thought to play a primary role in the initiation and maintenance of central

sensitization of nociceptive neurons.[11,40,41] In agreement with this notion, CFA-induced inflammation in the TMJ resulted in an increase in CGRP expression in the outer lamina of the STN at 2 hours that was sustained at days 7 and 21, the longest time point included in this study. CGRP receptors, which are present on TG neurons that provide sensory innervation of the TMJ capsule, are also expressed on second-order neurons and on the associated glial cells (astrocytes and microglia).[42]

To determine whether PKA was also involved in mediating the stimulatory effects of CFA, the PKA inhibitory molecule KT5720 (500 nM) was injected intrathecally immediately prior to bilateral CFA injection into the TMJ capsules. The concentration of 500 nM is lower than that used in other studies investigating the role of PKA in the central nervous system[43,44] and is not likely to cause inhibition of protein kinase G or protein kinase C (Ki > 2 μM). Administration of KT5720 significantly inhibited the sustained stimulatory effect of CFA on CGRP levels observed on days 7 and 21. Although KT5720 decreased the stimulatory effect of CFA at 2 hours, the level of repression did not reach significance. The coupling of CGRP receptor activation with PKA signal transduction in the STN is in agreement with a previous study reporting a similar relationship in the dorsal horn of the lower spinal cord.[24] Taken together, results from these cellular studies support the notion that CFA-induced inflammation in the TMJ involves a sustained increase in the expression of CGRP and the active form of PKA in the STN.

Activation of the PKA signaling pathway in spinal nociceptive neurons and glial cells is implicated in the development of a persistent state of central sensitization.[45–47] In response to CFA injection into the TMJ capsules, significantly elevated levels of PKA were observed for up to 21 days in neurons and microglia in the STN based on colocalization with NeuN and Iba1. Upon binding of CGRP to its receptor on primary neurons, adenylyl cyclase is activated and increases intracellular cAMP concentration, which leads to activation of PKA and stimulation of CGRP synthesis via an autocrine mechanism.[42] It was found that intrathecal administration of KT5720 inhibited CFA-mediated stimulation of PKA activation at each time point. PKA signaling leads to phosphorylation of the transcription factor CREB and its translocation into the nucleus, where it can bind to the cAMP response element DNA regulatory site located in the promoter region of the CGRP and numerous cytokine genes. Increased expression of these genes is implicated in promoting and sustaining a hyperexcitable state of spinal nociceptive neurons that involves glial activation.[11] Activation of astrocytes and microglia is known to contribute to prolonged sensitization of nociceptive neurons within the STN and the spinal cord, leading to development of chronic pain conditions.[10,48,49] Results from immunohistochemical studies of Iba1, which is implicated in microglia activation,[49] provided evidence that CFA-mediated inflammation in the TMJ promotes a sustained increase in Iba1 expression in the STN for up to 21 days postinjection. The CFA-mediated increase in Iba1 expression was greatly inhibited in response to intrathecal administration of the PKA inhibitor KT5720 at all time points. These results, in conjunction with the observed colocalization of activated PKA with Iba1 in the STN of CFA-only animals, provide evidence of a key role of PKA in the initial and sustained activation of microglia in response to CFA inflammation in the TMJ.

TMD pathology is characterized by the development of peripheral and central sensitization of trigeminal nociceptive neurons.[10] Having demonstrated an involvement of PKA in cellular events associated with central sensitization, the potential role of PKA signaling in nociceptive processing by trigeminal neurons in the STN was investigated. Intrathecal administration of KT5720 immediately prior to CFA injection into the TMJ capsules significantly reduced the average number of nocifensive head withdrawal responses to mechanical stimulation of the TMJ at each time point. Thus, inhibition of PKA signaling within the STN was sufficient to greatly reduce the nocifensive response associated with persistent inflammation within the TMJ. This finding is in agreement with results from a previous study that utilized KT5720 to demonstrate the involvement of PKA in mediating spinal nociceptive processes.[50] Intrathecal administration of KT5720 also inhibited CFA-induced increases in the nuclear expression of the signaling protein p-ERK in TG neurons. Translocation of p-ERK into neuronal nuclei is associated with increased expression of proinflammatory proteins that are known to promote peripheral nociceptor sensitization, including CGRP and cytokines.[51–53] Recent findings have provided evidence of bidirectional signaling in the trigeminal system, in which intrathecal administration of CGRP caused increased nuclear localization of p-ERK in the ganglion and increased neuron–satellite glial cell coupling.[54] Thus, blocking CFA-induced PKA activation in the STN, which resulted in lower CGRP levels, likely mediates in part the observed decrease in p-ERK and, hence, the inhibition of trigeminal nociception in response to mechanical stimulation.

A potential limitation of this study is that only male rats were utilized in a model of TMD, which is a condition reported to be more prevalent in females.[55,56] Future animal studies are planned to investigate sex differences in multiple models of TMD.

Conclusions

Results from this study provide evidence of the involvement of CGRP-PKA signaling in the STN, which promotes cellular changes associated with an increase in trigeminal nociception in a model of TMD. Inhibition of PKA signaling in the STN was shown to decrease the average number of nocifensive withdrawals to mechanical stimuli and was associated with decreased levels of nuclear p-ERK in TG neurons. These findings support the notion that inhibiting the PKA-mediated neuronal and glial changes to reduce nociception would be beneficial in the treatment of TMD.

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