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Inclusion of Cocoa as a Dietary Supplement Represses Expression of Inflammatory Proteins in Spinal Trigeminal Nucleus in Response to Chronic Trigeminal Nerve Stimulation

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Abstract

Scope—Central sensitization is implicated in the pathology of temporomandibular joint disorder (TMD) and other types of orofacial pain. We investigated the effects of dietary cocoa on expression of proteins involved in the development of central sensitization in the spinal trigeminal nucleus (STN) in response to inflammatory stimulation of trigeminal nerves.

Methods and results—Male Sprague Dawley rats were fed either a control diet or an isocaloric diet consisting of 10% cocoa powder 14 days prior to bilateral injection of complete Freund's adjuvant (CFA) into the temporomandibular joint to promote prolonged activation of trigeminal ganglion neurons and glia. While dietary cocoa stimulated basal expression of GLAST and MKP-1 when compared to animals on a normal diet, cocoa suppressed basal calcitonin gene-related peptide levels in the STN. CFA-stimulated levels of protein kinase A, $P2X_3$, P-p38, GFAP, and OX-42, whose elevated levels in the STN are implicated in central sensitization, were repressed to near control levels in animals on a cocoa enriched diet. Similarly, dietary cocoa repressed CFA-stimulated inflammatory cytokine expression.

Conclusion—Based on our findings, we speculate that cocoa enriched diets could be beneficial as a natural therapeutic option for TMD and other chronic orofacial pain conditions.

Keywords

Cocoa; Cytokines; Inflammation; Sensitization; Trigeminal

1 Introduction

Temporomandibular joint disorder (TMD) is the most common type of chronic orofacial pain referred to dentists [1–3]. TMD involve complex symptoms of pain and dysfunction of the masticatory system including the temporomandibular joint (TMJ), muscles of mastication, and adjacent tissues [4]. TMD is a major cause of chronic non-dental pain in the orofacial region, and hence negatively impacts quality of life. The underlying pathology of TMD involves sensitization and activation of the V3 or mandibular branch of the trigeminal nerve that provides sensory innervation to the muscles and joint, and functions to relay nociceptive signals to the spinal trigeminal nucleus (STN) [5, 6]. Sensitization of trigeminal neurons, characterized by a lowering of the activation threshold to chemical, thermal, or mechanical stimuli, is mediated by increased neuron-glia interactions in trigeminal ganglia

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Conflict of Interest
The authors declare that they have no potential conflict of interest.

and upper spinal cord [7–9]. Importantly, development of central sensitization, which involves second order neurons that transmit pain signals to the thalamus leading to a persistent pain state, is facilitated by hyperexcitable spinal astrocytes and microglia [10]. Under physiological conditions, these glial cells modulate neuronal excitability and maintain homeostasis by regulating the extracellular environment around neurons via the uptake of K^+ ions and glutamate by selective transporters [11, 12].

Regulation of the excitability state of neurons involved in pain transmission within the spinal cord is known to involve numerous proteins including the neuropeptide calcitonin gene-related peptide (CGRP) [13], the purinergic receptor $P2X_3$, cytokines, and proteins involved in signal transduction pathways [14]. CGRP stimulates glial cells in the spinal cord to release cytokines and other inflammatory molecules leading to a lower threshold of activation and contributing to persistent pain [15]. Increased levels of $P2X_3$ receptors on trigeminal neurons are indicative of central sensitization and increased pain signalling within the spinal cord [14, 16]. In addition, protein kinase A (PKA) promotes development of central sensitization by increasing the activity of glutamate receptors expressed on second order neurons that facilitate pain transmission [17–19]. Furthermore, cytokines are soluble proteins released from activated glial cells that directly sensitize nociceptors and increase neuronal sensitivity by increasing the expression of receptors and ion channels involved in pain and analgesia [20]. The mitogen-activated protein (MAP) kinases, which are a family of signal transduction enzymes activated in response to inflammatory stimuli, are also implicated in central sensitization [21]. The duration and magnitude of the MAP kinase response is regulated by MAP kinase phosphatases (MKP), which are enzymes involved in restoring elevated MAP kinase levels to normal via dephosphorylation [22, 23]. In particular, MKP-1 is a protein that preferentially dephosphorylates p38 and is reported to play a key role in the resolution of inflammation and pain [24, 25].

We have previously reported that inclusion of dietary cocoa was sufficient to suppress expression of proteins and signalling molecules in trigeminal ganglia associated with peripheral sensitization in response to TMJ inflammation [26]. There is emerging experimental evidence of the anti-inflammatory properties of cocoa that may help to explain the beneficial effects of cocoa in a variety of inflammatory ailments including insulin insensitivity and cardiovascular disease [27, 28]. Cocoa is obtained from the *Theobroma cacao* tree that is native to the tropical regions of the Americas. Historically, cocoa seeds were used in a variety of rituals by the Mayans and Aztecs who believed the seeds had medicinal benefits [29]. Persistent pathological pain, which is characteristic of TMD, is not effectively treated using standard pharmacological therapies. Therefore, the use of natural products offers a novel method for possibly preventing and treating chronic inflammatory diseases such as TMD [30, 31]. To date, most of the anti-inflammatory and anti-nociceptive effects attributed to compounds isolated from cocoa and other plants include the polyphenols catechins, anthocyanidins, and proanthocyanidins [32]. In addition, other bioactive compounds that are found abundantly in cocoa and reported to have anti-inflammatory properties, are phytosterols such as β-sitosterol [33]. The goal of this study was to extend our previous findings on cocoa to determine whether dietary cocoa could mediate cellular changes in the STN to suppress the development and maintenance of central sensitization in a chronic inflammatory model of TMD.

2 Materials and methods

2.1 Ethics statement

All animal studies were approved by the Institutional Animal Care and Use Committee at Missouri State University (Protocol 10004) and were conducted in compliance with all established guidelines in the Animal Welfare Act and National Institutes of Health. A

concerted effort was made to reduce the number of animals used and to minimize any suffering.

2.2 Animals and diet

Adult male Sprague-Dawley rats (200–230 g, Charles River Laboratories, Wilmington, MA) had unrestricted access to food and water and were kept on a 12-hour light/dark cycle. The temperature range was 21–23°C and relative humidity was between 40 and 55%. Changes in food and water consumption as well as weight and grooming behaviours were monitored and recorded daily to assess the overall health of the animals. All natural, non-alkalized davao cocoa powder (fat content of 18–20%) was purchased from Askinosie Chocolate (Springfield, MO) and incorporated into a diet based on a modified AIN-76A rodent diet (Research Diets, New Brunswick, NJ) containing 95.45 g cocoa powder/kg pellet weight [10% (g/g) or 6.7% of total energy intake] (Supporting Information Table S1). The polyphenolic content of the cocoa powder was 22.6 mg/g gallic acid equivalents as determined by the Folin-Ciocalteu assay [34]. All diets were isoenergetic and contained equal concentrations of carbohydrates, protein, and fat. Following a three-day acclimation period on the control diet (AIN-76A), rats were randomly placed into two groups receiving a normal control diet or cocoa enriched diet for 14 days. A total of 16 rats were used for all studies, which were equally split between four groups (control diet only, animals receiving cocoa enriched diets only, animals on control diets receiving an injection of complete Freund's adjuvant (CFA), and animals on cocoa enriched diets that received an injection of CFA). The same animals were used for immunohistochemistry and array analysis.

2.3 Chronic model of TMJ inflammation

Rats were anesthetized by inhalation of 3.5% isoflurane (VetEquip, Pleasanton, CA). After 14 days on the control diet or cocoa enriched diet, some rats received an injection of 50 μL CFA (1:1 in saline; Sigma-Aldrich, St. Louis, MO) in each TMJ capsule. All animals then continued their assigned diets for 7 more days before being sacrificed. The time of treatment, preparation, and amount of stimulatory agent were based on results from previous studies in our laboratory [26, 35]. As controls, some animals on each diet were not injected with CFA.

2.4 Tissue isolation and preparation

Spinal cord encompassing the spinomedullary junction (Vc/C1) transition zone containing the STN was removed from all rats following CO_2 asphyxiation and decapitation, trimmed at the obex and 5 mm posterior to the obex, and cut in half laterally along the dorsal ventral axis. A randomly chosen half of the tissue was quickly frozen in liquid nitrogen and used for cytokine array analysis. The other half of the tissue, which was used for immunohistochemistry, was placed in a solution of 4% paraformaldehyde overnight at 4°C. Following paraformaldehyde fixation, tissues were incubated in 15% sucrose in distilled water at 4°C for 1 hour and then 30% sucrose overnight at 4°C. Spinal cord tissues were positioned with the caudal side in contact with the slide, covered with OCT Compound (Sakura Finetek, Torrance, CA), and quickly frozen. Spinal cord sections (14 μm) containing the STN were sectioned transversely at a distance of 4–5 mm posterior to the obex using a cryostat set at −24°C (Microm HM 525, Thermo Scientific, Waltham, MA). All sections were mounted on Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA). To minimize variability, each slide used for immunohistochemistry contained 4 sections, one section from each experimental condition.

2.5 Immunohistochemistry

Slides containing sections of spinal cord were incubated in a solution of 0.1% Triton X-100 and 5% donkey serum (Jackson Immuno Research Laboratories, West Grove, PA) for 30 minutes. Next, sections were incubated with primary antibodies either for 3 hours at room temperature or overnight at 4°C and then with secondary antibodies for 1 hour at room temperature (Table 1). As a control, some sections were incubated with only secondary antibodies. Sections were mounted using Vectashield medium (H-1200) containing 4′, 6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) to co-stain cell nuclei. Images (200x) were collected using a Zeiss Axiocam mRm camera mounted on a Zeiss Imager Z1 fluorescent microscope equipped with an ApoTome. Image acquisition was performed using Zeiss AxioVision (Rel 4.8, Thornwood, NY).

2.6 Measurement of staining intensity

Images of the spinal cord tissue containing the STN were used for analysis. Three randomly chosen 200x images containing a similar number of cells as identified by DAPI, were analyzed for each experimental condition, which were repeated in 4 independent experiments, resulting in 16 images being analyzed for the intensity measurements. The relative staining intensity measurements were based on our previously published protocols [26, 36, 37]. Two researchers blinded to the experimental conditions independently performed the measurements. The staining intensity in spinal cord tissue was determined by measuring the mean gray intensity from four regions, in laminas I–III, of staining in the medullary horn and subtracting the intensity from acellular regions of the medullary horn. The fold-change in staining intensity was defined as the mean change in relative intensity in the experimental condition when compared to the mean of the unstimulated control tissue that was made equal to one.

2.7 Cytokine Array Analysis

The intracellular levels of 29 proteins were determined using the Rat Cytokine Array Panel A Array Kit (R&D Systems, Minneapolis MN) per manufacturer's instructions. Briefly, spinal cord tissue containing the STN (4–5 mm posterior to the obex) from each animal was sonicated in cell lysis solution (Bio-Rad, Madison, WI). Total protein levels in each sample were determined by the Bradford assay. Samples (200 μg) were diluted in Array Buffer 6 and Detection Antibody Cocktail and incubated on pre-blocked membrane arrays overnight at 4°C with gentle shaking. Membranes were then incubated in streptavadin conjugated peroxidase for 30 minutes and exposed to ECL Plus Detection Reagent (GE Healthcare, Pittsburgh, PA) for 5 minutes prior to developing on Kodak Biomax X-ray film (Sigma-Aldrich). Densitometric analysis was performed using ImageJ (Version 1.45, NIH, Bethesda, MD) using Gilles Carpentier's Protein Array Analysis plugin for ImageJ (Version 1.0, 2010) with rolling ball background subtraction. Spot data were normalized to the average of six positive control spots on each array. The fold-change in dot intensity was defined as the mean change in relative intensity in the experimental condition when compared to mean level of the unstimulated control tissue that was made equal to one.

2.8 Statistical analysis

Changes in immunostaining intensity and cytokine levels were evaluated using the Mann-Whitney U test. For all studies, results were considered significant when $p < 0.05$. All statistical tests were performed using SPSS (Version 16, IBM, Chicago, IL). Results are reported as mean ± SEM.

3 Results

3.1 Cocoa regulation of basal expression of GLAST; CGRP; and MKP-1 in the STN

While only minimal staining for the glial cell glutamate uptake transporter, GLAST, was observed in glia within the STN in naïve control animals, GLAST expression was increased in glial cells throughout the STN in animals receiving dietary cocoa (Fig. 1A). When compared to control animals (1.00 ± 0.06), cocoa treated animals exhibited a significant increase in staining intensity throughout the STN (2.12 ± 0.1, $p < 0.01$) (Fig. 1B).

In unstimulated control animals, CGRP immunoreactivity was readily detected in neurons located in a discrete band in laminas I and II within the STN (Fig. 2A). However, the expression of CGRP in animals consuming cocoa was decreased when compared to control levels. Inclusion of dietary cocoa caused a significant decrease in CGRP staining intensity (0.64 ± 0.09, $p < 0.01$) when compared to control animals (1.00 ± 0.12) (Fig. 2B).

A stimulatory effect was seen in the level of immunostaining for the MAP kinase phosphatase MKP-1. Expression of MKP-1 was increased in both neurons and glia in the STN in animals consuming cocoa when compared to levels in naïve control animals (Fig. 3A). While minimal staining was observed in control animals (1.00 ± 0.14), animals consuming cocoa exhibited a significant increase in staining intensity in neurons and glial cells (1.90 ± 0.12, $p < 0.01$) (Fig. 3B). Importantly, there were no observable differences in weight due to changes in food or water consumption nor were any noticeable differences in grooming or social behaviors in rats on the cocoa enriched diet when compared to rats fed control diets (data not shown).

3.2 CFA mediated elevation in the levels of $P2X_3$; PKA; and P-p38 are repressed by cocoa

Minimal expression of the ATP receptor $P2X_3$ was detectable in neurons within the STN obtained from naïve control animals, and in animals on a cocoa enriched diet (Fig. 4A). In contrast, the level of $P2X_3$ expression was increased in neurons in the STN in response to prolonged TMJ inflammation caused by injection of CFA into the joint capsule. Significantly, staining levels of $P2X_3$ were greatly repressed to near basal levels in animals consuming cocoa for 14 days prior to CFA injection. There was no significant difference in the staining intensity in animals eating cocoa alone (0.96 ± 0.07) when compared to levels in control animals (1.00 ± 0.09) (Fig. 4B). In contrast, CFA treatment caused a significant increase in $P2X_3$ staining intensity (1.82 ± 0.14, $p < 0.01$) that was repressed in animals consuming cocoa before CFA injection (1.09 ± 0.08, $p < 0.01$).

Normal immunostaining levels of the pro-inflammatory signalling marker PKA was observed in neurons and glia in the STN from naïve control animals and those consuming cocoa enriched diets (Fig. 5A). In contrast, PKA expression was increased 7 days after CFA injection in the TMJ. However, the stimulatory effect of CFA on PKA expression was greatly repressed in animals on a cocoa-enriched diet for 14 days prior to CFA injection. There was no significant difference in the staining intensity of rats treated with cocoa (1.07 ± 0.12) when compared to naïve control animals (1.00 ± 0.12) (Fig. 5B). In contrast, CFA caused a significant increase in staining intensity (1.97 ± 0.17, $p < 0.01$) over levels in control and cocoa animals. The stimulatory effect of CFA on PKA levels was significantly repressed in animals on a cocoa supplemented diet (1.36 ± 0.11, $p < 0.01$).

Minimal staining for the active form of the MAP kinase p38 (P-p38) was detected in neurons and glia within the STN obtained from naïve control animals and animals consuming cocoa (Fig. 6A). In contrast, P-p38 expression was increased in neurons and glia in the STN in response to prolonged TMJ inflammation caused by injection of CFA into the joint capsule. However, levels of P-p38 were repressed to near basal levels in animals eating

cocoa for 14 days prior to CFA injection. To identify the cell types exhibiting increased P-p38 expression, tissues were costained with antibodies directed against the neuronal protein NeuN, the microglial protein Iba1, or an astrocyte marker glial fibrillary acidic protein (GFAP). Based on our costaining results, elevated levels of P-p38 were only observed in neurons and microglia (data not shown). There was no significant difference in the staining intensity in animals eating cocoa alone (1.15 ± 0.19) when compared to levels in naïve control animals (1.00 ± 0.13) (Fig. 6B). In contrast, CFA treatment caused a significant increase in P-p38 staining intensity (1.74 ± 0.17, $p < 0.01$) that was repressed in animals consuming cocoa before injection of CFA (1.11 ± 0.15, $p < 0.01$).

3.3 Dietary cocoa represses CFA-induced expression of GFAP and OX-42 in the STN

Normal expression of GFAP, a marker of activated astrocytes, was detectable in the STN from naïve control animals and those consuming cocoa enriched diets (Fig. 7A). In contrast, GFAP expression was greatly stimulated in response to CFA injections. However, elevated staining levels of GFAP were greatly repressed in animals receiving cocoa for 14 days prior to CFA. There was no significant difference in the staining intensity of rats treated with cocoa (1.05 ± 0.13) when compared to control animals (1.00 ± 0.10) (Fig. 7B). In contrast, CFA caused a significant increase in staining intensity (1.92 ± 0.19, $p < 0.01$) over levels in control and cocoa-fed animals. The stimulatory effect of CFA on GFAP levels was significantly repressed in animals on a cocoa supplemented diet (1.20 ± 0.19, $p < 0.01$).

The effect of CFA and cocoa enriched diets on microglia within the STN was investigated using antibodies directed against OX-42, a marker of microglia activation. In tissues from naïve control or animals consuming cocoa, normal expression of OX-42 was observed in microglia (Fig. 8A). However, OX-42 expression was increased in microglia in the STN 7 days after CFA injection into the joint capsule. In contrast, animals pretreated with cocoa for 14 days before CFA injection exhibited much lower levels of OX-42 immunostaining when compared to animals that received CFA injections alone. There was no significant difference in the staining intensity in the STN of animals on a cocoa-enriched diet (1.04 ± 0.14) when compared to levels in naïve control animals (1.00 ± 0.13) (Fig. 8B). In contrast, CFA injection resulted in a significant increase in staining intensity (2.71 ± 0.21, $p < 0.01$) that was repressed in animals consuming cocoa prior to CFA injections (1.23 ± 0.14, $p < 0.01$).

3.4 CFA-mediated expression of cytokines is repressed by cocoa

To investigate the effects of cocoa on CFA-mediated increases on cytokine levels, sections of spinal cord containing the STN were used for protein array analysis. CFA stimulation caused a significant ($p < 0.05$) increase in 14 of the 29 proteins when compared to levels in control tissues obtained from naïve animals (Table 2). While seven proteins (CINC-2α/β, IL-1α, IL-2, IL-4, IL-13, LIX, and RANTES) were increased 2 to 2.4-fold, four proteins (IL-3, IL-17, IP-10, and MIG) were increased 2.5 to 3-fold and an additional three proteins (IL-1ra, TNF-α, and VEGF) were increased greater than 3-fold. However, the elevated levels of eight cytokines were significantly repressed in animals pretreated with cocoa for 14 days before CFA injection, while six cytokines were not appreciably changed by cocoa (Table 2). Animals receiving only cocoa supplemented diets had increases in two cytokines (IL-1ra and MIG) that were both increased 2 to 2.4-fold. A summary of the effect of cocoa on CFA mediated regulation of all 29 cytokines is provided as Supporting Information Table S2. Thus, dietary cocoa differentially regulates the expression of cytokines in the STN in response to prolonged TMJ inflammation.

4 Discussion

A main finding of our study was that inclusion of cocoa in the diet of Sprague-Dawley rats mediated cellular changes in the STN implicated in central sensitization in response to prolonged inflammation of the TMJ. The amount of cocoa used in our study was equivalent to daily consumption of 33 g of cocoa powder. The injection of CFA into the TMJ capsule, which causes prolonged tissue inflammation and pain mediated via sensitization and activation of trigeminal nerves, is utilized to mimic TMD pathology [38–42]. Excitation of trigeminal neurons results in the release of the neurotransmitter glutamate and neuromodulator CGRP from their terminals within the STN and subsequent activation of second order nociceptive neurons and spinal glia implicated in promoting central sensitization [43]. Importantly, we found that cocoa was able to increase the glial expression of GLAST, a protein that functions to remove the excitatory neurotransmitter glutamate from the external environment around second order neurons [11, 44]. GLAST is member of the excitatory amino acid transporters that function to maintain neuronal homeostasis by controlling the amount of excitatory amino acids localized in the extracellular space around neurons. Another novel finding from our study was that dietary cocoa was sufficient to suppress the expression of the neuropeptide CGRP within the STN. Elevated levels of CGRP within the spinal cord are associated with the development and maintenance of central sensitization [13]. CGRP release within the spinal cord is reported to increase activity of spinal glial cells leading to increased synthesis and secretion of pro-inflammatory molecules including cytokines. The changes in the levels of GLAST and CGRP caused by dietary cocoa would be expected to suppress neuron-glia interactions mediated by glutamate and CGRP in the STN that are implicated in persistent pain states.

In response to trigeminal neuron activation, CGRP release in the spinal cord promotes sensitization of nociceptive neurons via upregulation of the signal transduction protein PKA and $P2X_3$, a receptor associated with nociceptive signalling in neurons, and spinal glia [45–47]. We found that dietary cocoa could suppress the stimulatory effect of CFA-induced inflammation in the TMJ on PKA levels in neurons and glia in the STN. Elevated PKA levels stimulate presynaptic pain neurotransmitter release via phosphorylation of neuronal ion channels, transcription factors, and synaptic transport proteins [48, 49]. Another way that CGRP is implicated in the development of central sensitization is by promoting expression of $P2X_3$ receptors in trigeminal neurons [47, 50]. Elevated levels of ATP in the spinal cord and activation of the ionotropic $P2X_3$ receptors are associated with persistent pain conditions [51, 52]. In support of this notion, $P2X_3$ gene disruption in knockout animals and inhibition of $P2X_3$ receptors with selective antagonists results in reduced pain-related behaviour in inflammatory pain models [49, 51, 53–55]. These findings support an important role of $P2X_3$ in the development and maintenance of chronic pain signalling in sensory neurons. Based on our findings, dietary cocoa represses the prolonged CFA-mediated increases in PKA and $P2X_3$, two proteins known to play an important role in central sensitization.

Another important finding from our study is that dietary cocoa stimulates an increase in the expression of the anti-inflammatory protein MKP-1, while repressing the CFA-induced increase in the level of the active form of p38 in the STN. Increased levels of P-p38 mediate sensitization of primary and second order nociceptive neurons by upregulating ion channel expression and activity and membrane receptor expression [56, 57]. In addition, elevated P-p38 levels stimulate synthesis and secretion of cytokines from spinal glia that promote and maintain a hyperexcitable state of neurons [21, 56, 58, 59]. The cocoa-mediated repression of P-p38 observed in our study was likely due to the increased expression of MKP-1 in the STN since MKP-1 is reported to preferentially suppress active, phosphorylated p38 levels in response to inflammatory stimuli or nerve injury [24]. The activity of MAP kinases is

regulated by the family of proteins known as MAP kinase phosphatases that are induced in response to inflammatory stimuli, and thus, function in a compensatory manner to restore normal levels of active MAP kinases [25]. Importantly, data from recent studies provide evidence that mice lacking the MKP-1 gene have elevated MAP kinase activity [60], increased cytokine-induced inflammation [61], and higher susceptibility to inflammatory injuries [23]. Thus, MKPs perform an important function by modulating nociceptive responses to inflammatory stimuli and restoring cellular homeostasis. Interestingly, the cocoa-induced increase in levels of MKP-1 may be partly responsible for the lower levels of CGRP in the STN since CGRP gene expression is regulated by MAP kinases [62, 63].

In this study, we provide evidence that cocoa can suppress the activation of both astrocytes and microglia in the STN, and repress elevated levels of cytokines caused by prolonged joint inflammation. In response to persistent CFA-induced TMJ inflammation, trigeminal neurons are known to release inflammatory mediators that cause activation of spinal glial cells [10]. In agreement with previous studies, we found that immunoreactive levels of GFAP, which is used as a marker of activated astrocytes, and OX-42, a biomarker of activated microglia, were significantly elevated in response to CFA injection into the TMJ [6, 41]. It is now generally accepted that hyperactivation of spinal glia, in particular astrocytes and microglia, contribute to the development and maintenance of inflammatory pain [41, 44, 64]. These cells are thought to promote and sustain inflammatory pain by the release of cytokines that act directly to sensitize nociceptors and increase neuronal sensitivity to chemical, thermal, and mechanical stimuli by increasing the expression of receptors and ion channels involved in pain and analgesia [20, 65]. Interestingly, we found that cocoa, provided as 10% of the total solid diet, repressed the level of many but not all cytokines to baseline levels. Thus, it appears that cocoa functions to modulate the level of these inflammatory mediators but does not completely suppress the inflammatory response following CFA injection in the TMJ. Based on our data, we propose that dietary cocoa can function to inhibit neuron-glia interactions and cytokine release and therefore prevent key cellular events known to promote and maintain central sensitization in response to TMJ inflammation.

Our findings support an important role of dietary cocoa to suppress pathological inflammatory events associated with neurological diseases, cardiovascular disease, diabetes, arthritis, and cancer [66–68]. Our observed changes in protein expression within the upper spinal cord are likely the result of direct effects of cocoa metabolites since dietary plant flavonoids and sterols can cross the blood brain barrier, accumulate in the brain, and modulate neural function [69, 70]. The inhibitory effects of cocoa on nociceptive neurons and glial cells in the spinal trigeminal nucleus are likely mediated by multiple secondary plant metabolites found in cocoa [32, 66]. In support of this notion, flavonoids have been reported to attenuate microglial activation and suppress cytokine release likely by a mechanisms involving inhibition of pro-inflammatory NF-kB and MAP kinase pathways [71, 72]. Flavonoids are also reported to function by preserving cognitive abilities in aging rats and inhibiting programmed neuronal cell death or apoptosis [73, 74]. In addition, there is evidence from multiple studies of the anti-inflammatory effects of β-sitosterol [33, 75, 76]. In summary, there is emerging evidence that plant metabolites from cocoa are neural protective since dietary cocoa has been shown to repress neuronal sensitization of nociceptive neurons, inhibit neuroinflammation, and suppress neurodegeneration.

The findings of this study are in agreement with results from our previous study that provided evidence that dietary cocoa mediated suppression of neuron and glial activity associated with peripheral sensitization of trigeminal nociceptors in response to chronic inflammation [26]. Taken together results from this study and our previous study provide evidence that inclusion of cocoa in the diet of rats is sufficient to modulate expression of proteins implicated in the development of peripheral sensitization and central sensitization,

which are pathophysiologic events implicated in TMD pathology. The development of chronic pain, as reported in patients with TMD, is an increasing burden for our society. TMD affects as many as 15% of the adult population [2, 4], is more prevalent in women than men, and is highest during the reproductive years [77]. Given the significant health impact of this disease, it is imperative that treatments are identified. However, orofacial pain diseases such as TMD that are associated with inflammation and pain are reported to be some of the most debilitating human conditions and most difficult to treat effectively [78–80]. To date, there are no FDA approved pharmacological therapies for TMD. While many orofacial pain patients treat their inflammatory pain with nonsteroidal anti-inflammatory drugs (NSAIDs), COX-2 inhibitors, and opioids, the prolonged use of all of these treatments is limited due to the occurrence of side effects [81, 82]. Based on our findings, we propose that regular consumption of cocoa would protect against the development of persistent peripheral and central trigeminal sensitization that can lead to potential pathological responses caused by prolonged joint inflammation. Furthermore, we speculate that dietary cocoa would provide a nutraceutical means for repressing the development of central sensitization, a key component of inflammatory pain, and thus be useful in the treatment of TMD and possibly other chronic orofacial pain conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

CGRP	calcitonin gene-related peptide
CFA	complete Freund’s adjuvant
GFAP	glial fibrillary-associated protein
GLAST	glutamate-aspartate transporter
PKA	protein kinase A
MAP	mitogen-activated kinase
MKP	MAP kinase phosphatase
STN	spinal trigeminal nucleus
TMD	temporomandibular disorder
TMJ	temporomandibular joint

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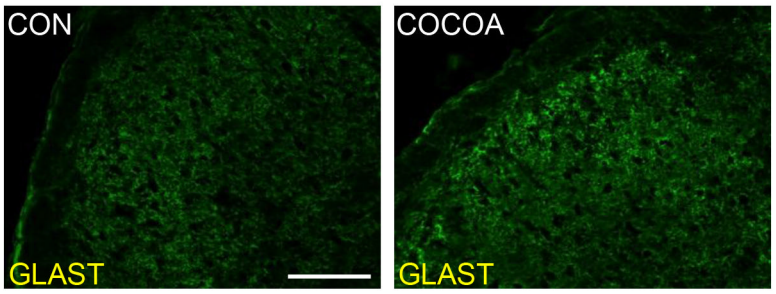
A



B

	CON	COCOA
GLAST	1.00 ± 0.06	2.12 ± 0.10*

Figure 1.

Cocoa enriched diets increase basal GLAST expression in spinal glial cells. Sections of spinal cord containing the STN were obtained from untreated animals (CON) and animals on a 10% cocoa enriched diet for 14 days (COCOA). (A) Representative images of spinal cord containing the STN stained for GLAST are shown. (B) The average fold-change ± SEM of GLAST staining intensity when compared to the mean of the control that was made equal to one is reported ($n = 3$ independent experiments). * $p < 0.01$ increase when compared to control. Magnification bar = 50 μm.

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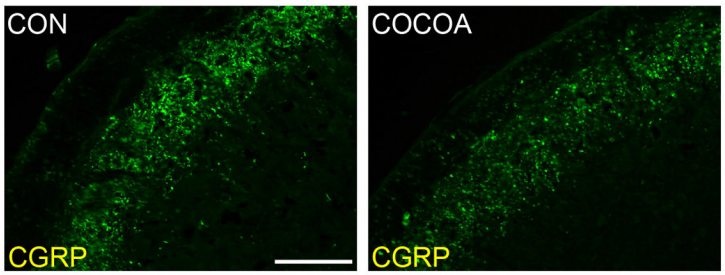

B

	CON	COCOA
CGRP	1.00 ± 0.12	0.64 ± 0.09*

Figure 2.

Basal CGRP expression in the STN is suppressed by cocoa- enriched diet. Spinal cord sections containing the STN were obtained from untreated animals (CON) and animals on a 10% cocoa-enriched diet (COCOA). (A) Representative images of spinal cord containing the STN stained for CGRP are shown. (B) The average fold-change ± SEM of CGRP staining intensity when compared to the mean of the control that was made equal to one is reported ($n = 3$). * $p < 0.01$ decrease when compared to control. Magnification bar = 50 μm.

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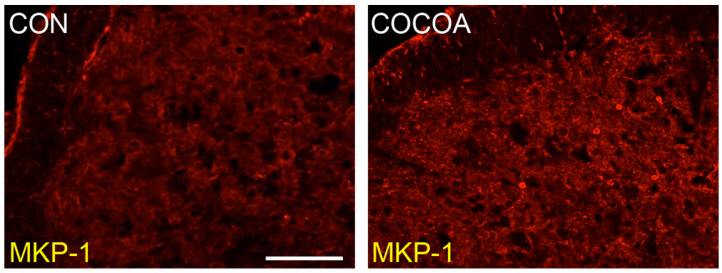

B

	CON	COCOA
MKP-1	1.00 ± 0.14	1.90 ± 0.12*

Figure 3.

Basal expression of MKP-1 is increased in neurons and glia in the STN of rats on cocoa supplemented diets. (A) Representative images of the STN region in spinal cord obtained from control animals (CON) or animals consuming cocoa stained for MKP-1 are shown. (B) The average fold-change ± SEM of MKP-1 staining intensity when compared to the mean of the control that was made equal to one is reported ($n = 3$). * $p < 0.01$ increase when compared to control. Magnification bar = 50 μm.

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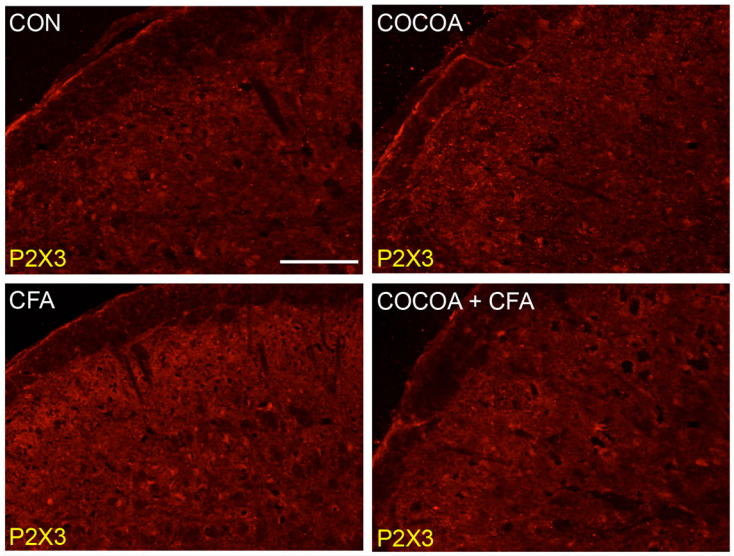

B

	CON	COCOA	CFA	COCOA + CFA
P2X3	1.00 ± 0.09	0.96 ± 0.07	1.82 ± 0.14*	1.09 ± 0.08#

Figure 4.

CFA-mediated upregulation of $P2X_3$ expression in STN neurons is repressed in rats receiving cocoa as a dietary supplement. Sections of spinal cord containing STN were obtained from untreated animals (CON), animals on diets supplemented with 10% cocoa for 14 days (COCOA), animals 7 days post CFA injection (CFA), or animals on a cocoa-enriched diet and injected with CFA 7 days prior to harvesting the spinal cord (COCOA + CFA). (A) Images of the STN stained for $P2X_3$ are shown. (B) The average fold change ± SEM of $P2X_3$ staining intensity when compared to the mean of the control that was made equal to one is reported ($n = 3$). * $p < 0.01$ increase when compared to control while # $p < 0.01$ decrease compared to CFA. Magnification bar = 50 μm.

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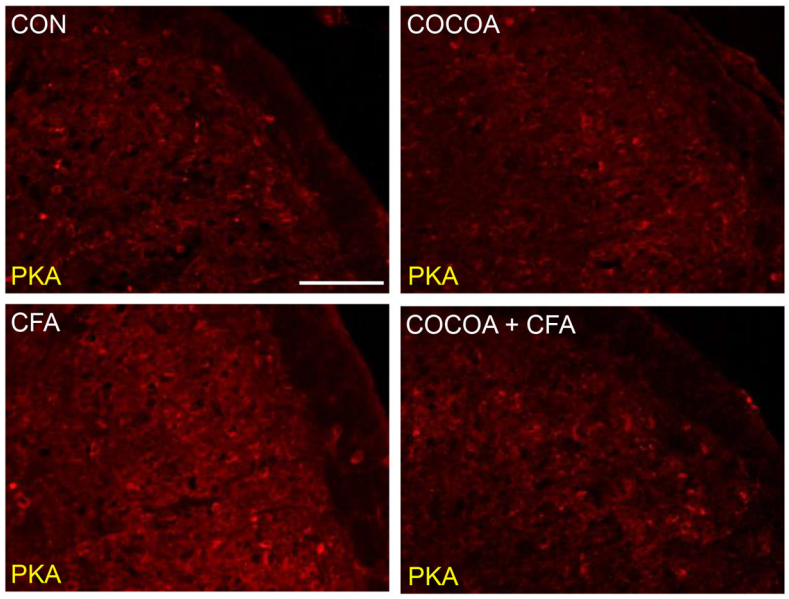

B

	CON	COCOA	CFA	COCOA + CFA
PKA	1.00 ± 0.12	1.07 ± 0.12	1.97 ± 0.17*	1.36 ± 0.11‡

Figure 5.

Stimulated PKA expression in STN neurons and glia is repressed by dietary cocoa. (A) Representative images of the STN immunostained for PKA are shown. Tissues were obtained from control animals (CON) or animals on 10% cocoa enriched diets for 14 days (COCOA), animals injected with CFA for 7 days (CFA), or animals on a 10% cocoa diet for 14 days then injected with CFA for 7 days prior to harvesting the spinal cord (COCOA + CFA). (B) The average fold change ± SEM of PKA staining intensity when compared to the mean of the control that was made equal to one is reported ($n = 3$).* $p < 0.01$ increase when compared to control while ‡ $p < 0.01$ decrease from CFA and $p < 0.01$ increase from control. Magnification bar = 50 μm.

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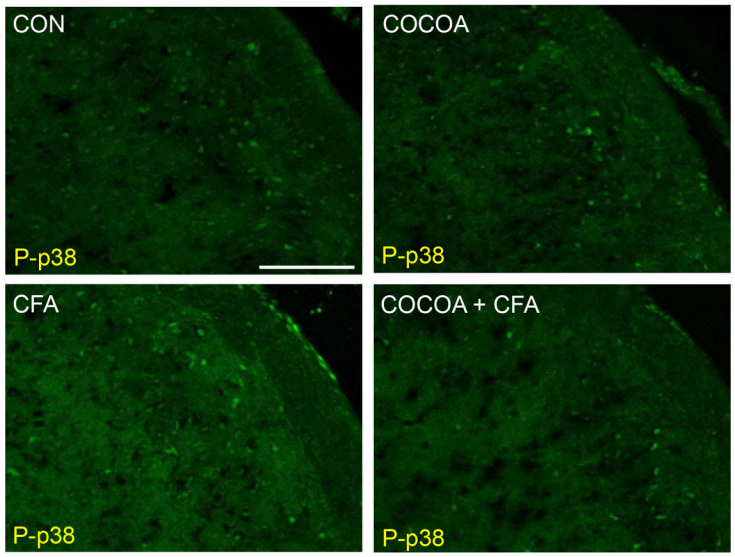

B

	CON	COCOA	CFA	COCOA + CFA
P-p38	1.00 ± 0.13	1.15 ± 0.19	1.74 ± 0.17*	1.11 ± 0.15#

Figure 6.

Increased expression of P-p38 in STN neurons and microglia in response to CFA is repressed by cocoa. (A) Representative images of the STN immunostained for P-p38 are shown. Tissues were obtained from control animals (CON) or animals on 10% cocoa enriched diets for 14 days (COCOA), animals injected with CFA for 7 days (CFA), or animals on a 10% cocoa diet for 14 days then injected with CFA for 7 days prior to harvesting the spinal cord (COCOA + CFA). (B) The average fold change ± SEM of P-p38 staining intensity when compared to the mean of the control that was made equal to one is reported ($n = 3$). * $p < 0.01$ increase when compared to control while # $p < 0.01$ decrease from CFA. Magnification bar = 50 μm.

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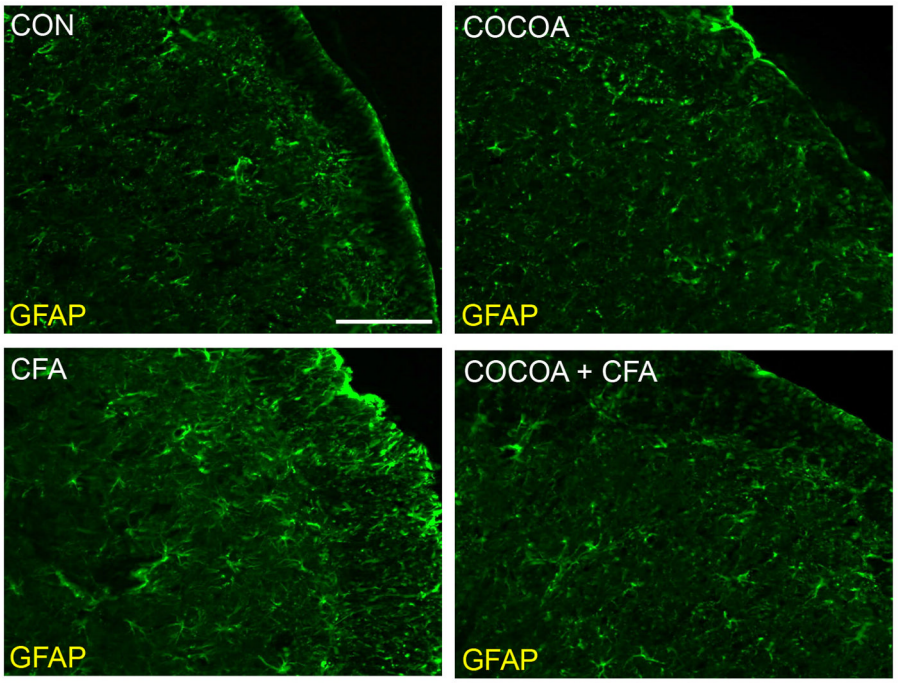

B

	CON	COCOA	CFA	COCOA + CFA
GFAP	1.00 ± 0.10	1.05 ± 0.13	1.92± 0.19*	1.20 ± 0.19#

Figure 7.

Cocoa represses CFA mediated upregualtion of GFAP expression in STN astrocytes. (A) Representative images of the STN immunostained for GFAP are shown. Tissues were obtained from control animals (CON) or animals on 10% cocoa enriched diets for 14 days (COCOA), animals injected with CFA for 7 days (CFA), or animals on a 10% cocoa diet for 14 days then injected with CFA for 7 days prior to harvesting the spinal cord (COCOA + CFA). (B) The average fold change ± SEM of GFAP staining intensity when compared to the mean of the control that was made equal to one is reported ($n = 3$). * $p < 0.01$ increase when compared to control while # $p < 0.01$ decrease from CFA. Magnification bar = 50 μm.

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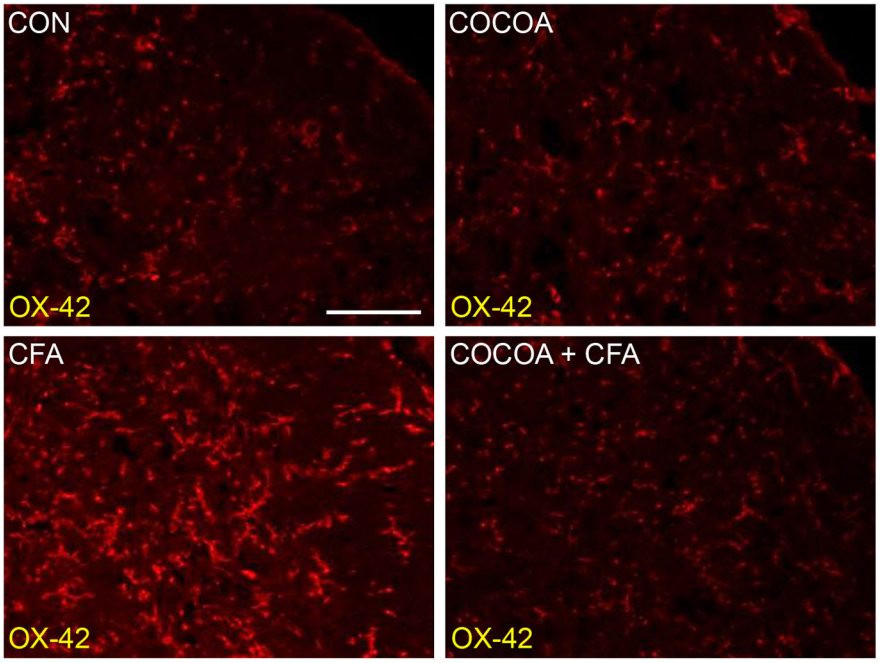

B

	CON	COCOA	CFA	COCOA + CFA
OX-42	1.00 ± 0.13	1.04 ± 0.14	2.71 ± 0.21*	1.23 ± 0.14#

Figure 8.

CFA stimulation of OX-42 expression in STN microglia is repressed in rats receiving cocoa supplemented diets. (A) Representative images of the STN immunostained for OX-42 are shown. Tissues were obtained from control animals (CON) or animals on 10% cocoa enriched diets for 14 days (COCOA), animals injected with CFA for 7 days (CFA), or animals on a 10% cocoa diet for 14 days then injected with CFA for 7 days prior to harvesting the spinal cord (COCOA + CFA). (B) The average fold change ± SEM of OX-42 staining intensity when compared to the mean of the control that was made equal to one is reported ($n = 3$). * $p < 0.01$ increase when compared to control while # $p < 0.01$ decrease from CFA. Magnification bar = 50 μm.

Table 1

Summary of Antibodies Used for Immunohistochemistry

Antibody	Host	Supplier	Dilution
GLAST	Rabbit	Abcam	1:200 3 hours
CGRP	Rabbit	Sigma	1:1000 3 hours
MKP1	Goat	Everest	1:500 3 hours
P2X3	Rabbit	Thermosci	1:1000 3 hours
PKA	Rabbit	Epitomics	1:500 3 hours
P-p38	Rabbit	Cell Signaling	1:200 overnight
GFAP	Rabbit	Dako	1:1000 30 minutes
OX-42	Mouse	Abcam	1:200 overnight
NeuN	Mouse	Millipore	1:1000 3 hours
Iba-1	Rabbit	Wako	1:400 3 hours
Alexa 488	Rabbit, Mouse	Invitrogen	1:500 1 hour
Alexa 594	Rabbit, Mouse, Goat	Invitrogen	1:500 1 hour

Table 2

Relative Fold-Change in Cytokine Levels in the Spinal Trigeminal Nucleus in Response to CFA and Cocoa Enriched Diets. The level of all cytokines were significantly ($p < 0.05$) elevated above control levels.

	Fold-Change From Control	
	CFA	**COCOA +CFA**
CINC-2α/β	2.26 ± 0.86	1.26 ± 0.69#
IL-1α	2.00 ± 0.56	1.58 ± 0.82#
IL-1ra	4.69 ± 2.31	3.05 ± 0.80
IL-2	2.33 ± 0.60	2.33 ± 0.73
IL-3	2.58 ± 0.78	2.30 ± 0.44
IL-4	2.20 ± 0.76	1.36 ± 0.44#
IL-13	2.04 ± 0.46	1.25 ± 0.57#
IL-17	2.83 ± 0.64	1.61 ± 0.62#
IP-10	2.72 ± 0.72	3.12 ± 0.83
LIX	2.41 ± 0.68	2.38 ± 0.81
MIG	2.94 ± 1.35	2.30 ± 0.74
RANTES	2.42 ± 0.72	1.83 ± 0.49#
TNF-α	3.23 ± 1.41	1.75 ± 0.90#
VEGF	4.27 ±1.71	2.57 ± 0.86#

#designates when $p < 0.05$ when compared to CFA simulation.