IBRO Neuroscience Reports 13 (2022) 187–197

Contents lists available at ScienceDirect

IBRO Neuroscience Reports

journal homepage: www.sciencedirect.com/journal/IBRO-Neuroscience-Reports

Research paper

Grape seed extract suppresses calcitonin gene-related peptide secretion and upregulates expression of GAD 65/67 and GABAB receptor in primary trigeminal ganglion cultures

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ARTICLE INFO

Keywords:
Trigeminal ganglion
Calcitonin gene-related peptide
GABA
GABA receptors
Satellite glia
Schwann cells
Grape seed extract

ABSTRACT

The trigeminal ganglion is implicated in the underlying pathology of migraine and temporomandibular joint disorders (TMD), which are orofacial pain conditions involving peripheral and central sensitization. The neuropeptide calcitonin gene-related peptide (CGRP) is synthesized in some trigeminal ganglion neurons, and its release promotes inflammation, peripheral and central sensitization, and pain signaling. Recent studies in preclinical migraine and TMD models provide evidence that dietary supplementation with grape seed extract (GSE) inhibits trigeminal pain signaling. The goal of this study was to investigate the cellular mechanisms by which GSE modulates primary trigeminal ganglion cultures. The effect of GSE on CGRP secretion was determined by radioimmunoassay. To determine if GSE effects involved modulation of CGRP or the GABAergic system, expression of CGRP, GAD 65 and 67, GABAA receptor, and GABAB1 and GABAB2 receptor subunits were investigated by immunocytochemistry. GSE significantly inhibited basal CGRP secretion but did not alter neuronal CGRP expression. GAD 65 and 67 expression levels in neurons were significantly increased in response to GSE. While GSE did not cause a change in the neuronal expression of GABAA, GSE significantly increased GABAB1 expression in neurons, satellite glial cells, and Schwann cells. GABAB2 expression was significantly elevated in satellite glia and Schwann cells. These findings support the notion that GSE inhibition of basal CGRP secretion involves increased neuronal GAD 65 and 67 and GABAB receptor expression. GSE repression of CGRP release coupled with increased GABAB1 and GABAB2 glial cell expression would be neuroprotective by suppressing neuronal and glial excitability in the trigeminal ganglion.

1. Introduction

The development of peripheral and central sensitization and pain signaling associated with prevalent, debilitating diseases such as migraine and temporomandibular joint disorder is mediated by trigeminal ganglion neurons (Sessle, 2011; Chichorro et al., 2017; Su and Yu, 2018). The trigeminal ganglion is comprised of three main branches that provide sensory innervation to most of the head and face and is the pathway for transmission of painful stimuli from the peripheral tissues to the ganglion and central nervous system (Shankland, 2000). In response to ischemia or tissue injury, the neuropeptide calcitonin gene-related peptide (CGRP) is released from the terminals of trigeminal Aδ and C fibers to promote an inflammatory response and initiate pain signaling (Iyengar et al., 2017). Elevated levels of CGRP are implicated in migraine and TMD pathology (Kopp, 2001; Tso and Goadsby, 2017). CGRP binding to receptors on the satellite glial cells and Schwann cells promotes initiation and maintenance of an inflammatory loop within the ganglion that lowers the activation threshold of neurons resulting in peripheral sensitization and enhanced pain signaling (Durham, 2016; Messlinger et al., 2020).

Previous findings from our laboratory have provided evidence that the addition of a commercially available grape seed extract (GSE) as a daily supplement to the drinking water of Sprague-Dawley rats inhibits pain signaling in preclinical models of TMD and migraine (Cornelison et al., 2020, 2021; Woodman et al., 2022). Furthermore, GSE inhibited the development of central sensitization within the spinal cord and peripheral sensitization in the trigeminal ganglion by modulating the excitability state of neurons and glia (Cady et al., 2010; Cornelison et al., 2020, 2021; Woodman et al., 2022). The inhibitory effect of GSE involves repression of basal CGRP expression in the spinal cord and

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https://doi.org/10.1016/j.ibneur.2022.08.006
Received 8 July 2022; Accepted 20 August 2022
Available online 24 August 2022

involved in part, activation of gamma-aminobutyric acid (GABA) receptors within the spinal cord (Cornelison et al., 2020). The modulatory effects of the neurotransmitter GABA, which is synthesized in neurons by the enzymes glutamate decarboxylase (GAD) 65 and 67, are mediated via activation of the GABA receptors GABAA and GABAB (Barker and Hines, 2020; Evenseth et al., 2020). GAD 65 is localized to the plasma membrane of GABAergic neurons, while GAD 67 is found primarily in the cytoplasm of neurons. Both GAD isoforms are abundantly expressed in trigeminal ganglion neurons (Watanabe et al., 2002; Vit et al., 2009). Although GSE was shown to involve modulation of CGRP and activation of GABAB receptors in the spinal cord (Cady et al., 2010; Cornelison et al., 2021), the effects of GSE on trigeminal ganglion neurons and glial cells that express GABAB and GABAA receptors is not known.

GABAB, a G-protein coupled receptor comprised of the subunits GABAB1 and GABAB2 (Jones et al., 1998), is expressed on the cell surface of trigeminal ganglion neurons (Vit et al., 2009; Takeda et al., 2013), satellite glia (Takeda et al., 2015), and Schwann cells (Magnaghi et al., 2004). The GABAB receptor functions as a heterodimer with GABAB1 being the subunit that binds GABA while GABAB2 facilitates translocation of GABAB1 to the cell surface, stabilization of the receptor, and coupling to the inhibitory $G_{i/o}$ protein. Activation of the GABAB receptor would inhibit activity of the signal transduction enzyme adenylate cyclase, which is positively regulated by CGRP (Russell et al., 2014). The inhibition of adenylate cyclase would cause a reduction in levels of the secondary messenger cyclic AMP (cAMP) within the cell, resulting in suppression of signal transmission, secretion, and neuronal excitability. In addition, activation of the GABAB receptor would mediate activation of inwardly rectifying K^+ (GIRK) channels and inhibit voltage-gated calcium channels within neurons (Bowery et al., 2002; Padgett and Slesinger, 2010; Terunuma, 2018). Similarly, activation of the GABAB receptor expressed on satellite glial cells was shown to stimulate K_{ir} channels to suppress excitability (Takeda et al., 2015). GABAA is an ionotropic or ligand-gated ion channel receptor and facilitates an influx of negatively charged chloride ions (Mihic and Harris, 1997) that inhibit generation of an action potential and the release of neurotransmitters and neuropeptides, as well as other pro-inflammatory molecules. Hence, activation of these GABA receptor-mediated mechanisms that lower the excitability state of neurons and glia would function in a neuroprotective manner to suppress CGRP-mediated development of peripheral sensitization.

Given the prevalence, significant morbidity, and major social and economic ramifications of chronic orofacial pain conditions including TMD and migraine (List and Jensen, 2017; Burch et al., 2018), there is a need for effective and safe preventative therapeutics. Hence, identification of novel alternatives to pharmaceutical drugs to modulate inflammation and pain is warranted. The use of natural products found in plants provides the potential for managing inflammatory responses and pain signaling. The goal of this study was to investigate the cellular mechanisms by which the proanthocyanin-enriched Healthy Origins MegaNatural® BP-Grape Seed Extract modulates neuron and glial cell function using primary trigeminal ganglion cultures. Results from this study provide evidence that GSE inhibits basal CGRP secretion, increases neuronal GAD levels, and stimulates neuronal and glial GABAB expression, and as such likely functions in a neuroprotective role in the trigeminal ganglion.

2. Material and methods

2.1. Animals

Animal protocols were approved by Missouri State University's Institutional Animal Care and Use Committee and conducted in compliance with all established procedures in the Animal Welfare Act, National Institutes of Health, and ARRIVE Guidelines. A concerted effort was made to minimize suffering, as well as the number of animals used in this study. Adult pregnant female Sprague-Dawley rats were purchased from Missouri State University's internal breeding colonies at the Jordan Valley Innovation Center (Springfield, MO). Animals were housed in clean, plastic cages with unlimited access to food and water. The holding room was kept at a constant temperature of 22–24 °C, with a 12-hour light/dark cycle. Three to five-day-old male and female neonatal pups were used to establish trigeminal ganglion cultures.

2.1.1. Establishment of primary trigeminal ganglion cultures

Primary cultures of trigeminal ganglia were established based on our previously published protocols (Durham and Russo, 1999; Bowen et al., 2006; Abbey et al., 2008; Vause and Durham, 2009; Durham and Masterson, 2013). Briefly, trigeminal ganglia obtained following decapitation of 3–5-day-old male and female neonatal Sprague-Dawley rats were placed in ice cold Leibovitz (L-15, Sigma-Aldrich) plating media immediately following dissection. Tissues were incubated in L-15 media containing 10 mg/mL Dispase II (Sigma-Aldrich) and 1 unit/μL RQ1 RNase-free DNase (Promega, Madison, WI) and rotated at 15 RPM in a 37 °C incubator for 30 min. Following digestion, tissues were centrifuged at 500 RPM for 2 min and resuspended in 5 mL of L-15 plating media. Tissues were dissociated by vigorous mechanical trituration and the supernatant containing cells transferred to 15 mL tubes prior to being centrifuged at 1300 RPM for 3 min to pellet neurons and glia. Isolated cells were resuspended in 37°C L-15 medium containing 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA), 50 mM glucose (Sigma-Aldrich), 250 mM ascorbic acid (Sigma-Aldrich), 8 mM glutathione (Sigma-Aldrich), 2 mM glutamine (Sigma-Aldrich), and 10 ng/mL mouse 2.5S nerve growth factor (Alomone Laboratories, Jerusalem, Israel). An antibiotic mixture of penicillin (100 units/mL) and streptomycin (100 μg/mL, Sigma-Aldrich), and the antimycotic amphotericin B (2.5 mg/mL, Sigma-Aldrich) was added to the supplemented L15 media, which is referred to as TG complete medium.

For immunocytochemistry studies, cells isolated from three trigeminal ganglia were plated on 24 Poly-D-Lysine coated glass coverslips (Electron Microscopy Sciences, Hatfield, PA) in 300 μL of media that were placed in a 24-well plate (Greiner Bio-One, Monroe, NC) and incubated at 37 °C. For the secretion studies, cells isolated from 30 trigeminal ganglia were plated directly into wells of a 24-well tissue culture plate (Corning Incorporated-Life Sciences, Durham, NC) in 250 μL of media and incubated at 37 °C. For the toxicity studies, cells isolated from 12 trigeminal ganglia were plated directly into wells of a 96-well tissue culture plate (Midwest Scientific, Fenton, MO) in 200 μL of media and incubated at 37 °C. In all studies, some cells were left untreated and served as naïve controls while other cells were incubated with a 1:10,000 dilution of a 5 mg/mL stock solution (final concentration of 0.5 ng/μL) of MegaNatural®-BP Grape Seed Extract (GSE, Healthy Origins, Pittsburgh, PA) prepared in cell water (BioWhittaker, Walkersville, MD). All cultures used for secretion, immunostaining, and toxicity studies were incubated for a total of 2 days at 37 °C.

2.1.2. Testing effect of GSE on cell viability

Cell viability of primary cultures incubated overnight with GSE at 0.5 ng/μL final concentration was evaluated in triplicate using a CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega) according to manufacturer's instructions. DMSO (10%) was used as a control. Briefly, 20 μL of CellTiter 96® Reagent was added to each well and incubated for 4 h at 37 °C. After incubation, the absorbance was measured at 490 nm using a microplate spectrophotometer. Following subtraction of background absorbance, average sample values were reported as absorbance at 490 nm.

2.1.3. GSE regulation of CGRP secretion

Primary cultures maintained in a 24-well tissue culture treated plate for 2 days were rinsed with 250 μL of phosphate-buffered saline (PBS, Sigma-Aldrich) prior to addition of 250 μL of HEPES-buffered saline (HBS: 22.5 mM HEPES, 135 mM NaCl, 3.5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 3.3 mM glucose, and 0.1% bovine serum albumin, at pH 7.4).

To determine basal secretion levels, cells with fresh prewarmed HBS were left to incubate at 37 °C for 1 h before media was placed into a microcentrifuge tube. This collected sample represents the "basal" levels of CGRP secretion. Fresh prewarmed HBS (250 μL) was then added to each of the wells. Some cells remained untreated (naïve), while other cells were incubated for 60 min at 37 °C with a final concentration of 0.5 ng/μL GSE. After 1 h, media from each well was placed into a microcentrifuge tube. This collected sample represents the "final" levels of CGRP secretion. Cells remaining in the wells were rinsed with PBS twice before being scraped and stored in microcentrifuge tubes for protein quantification. Basal and final media samples and cell pellets were stored at − 20°C.

The amount of CGRP secreted into the media before and after GSE incubation was measured using a radioimmunoassay specific for rat CGRP (Phoenix Pharmaceuticals, Inc., Burlingame, CA) following manufacturer's instructions and as described previously (Durham and Masterson, 2013). Radioactive counts for each sample were normalized to total protein concentration in each well, which was determined by the Bradford method (Bio-Rad Laboratories, Inc., Hercules, CA). Average means of CGRP secretion from naïve and GSE incubated samples from a minimum of 6 independent experiments performed in duplicate were determined and reported as pg CGRP/mg protein.

2.1.4. GSE modulation of protein expression by immunocytochemistry

Immunocytochemistry was utilized to investigate changes in protein expression levels of naïve and GSE incubated primary trigeminal ganglion cultures. One day after plating, primary cultures were incubated overnight with a 0.5 ng/μL GSE solution. The next day, cells were fixed with 4% paraformaldehyde in PBS, rinsed with PBS, and then incubated in a PBS solution containing 5% donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA) and 0.1% Triton (Sigma-Aldrich) for 20 min. After rinsing with PBS three times, cells were incubated at room temperature with primary antibody diluted in 5% donkey serum for three hours (Table 1). After primary incubation, cells were rinsed with PBS/Tween 80 solution (Fisher Scientific, Waltham, MA) and then PBS before being incubated with Alexa-Fluor conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). All secondary antibodies were prepared at a 1:200 dilution in PBS containing 5% donkey serum and incubated for one hour at room temperature. Cells were rinsed with PBS/Tween and then only PBS. Coverslips containing cells from the different experimental conditions were placed onto a double-frosted Fisher Scientific glass microscope slide and mounted using 70 μL of Vectashield anti-fade medium containing the nuclear fluorescent dye 4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA). Slides were covered with a glass coverslip and secured with a clear coat of nail polish.

All slides were stored at 4 °C and imaged within one week using a Zeiss Axiocam mRm camera (Carl Zeiss, Thornwood, NY) mounted on a Zeiss Imager Z1 fluorescent microscope. A minimum of three fluorescent 200x images were taken of each coverslip to study protein expression. Zen 2 software (Carl Zeiss) was used to adjust the image backgrounds to match the nonspecific intensities. ImageJ software was used to analyze the immunostaining intensity in each cell. Following subtraction of the average background, the average means of fluorescent intensities for each protein of interest in neurons and glial cells were determined and reported as average fold change ± SEM relative to the naïve average, which was set to one. Each condition was repeated in triplicate in a minimum of 6 independent experiments. A differential interference contrast filter was used in combination with DAPI staining for identification of cell types and morphology.

Table 1
Summary of antibodies used for immunocytochemistry.

Protein	Company; Catalog Number	Dilution
CGRP	Abcam; Ab36001	1:2000
β-tubulin	Abcam; Ab7751	1:5000
GAD 65/67	Abcam; Ab11070	1:1000
GABA A	Abcam; Ab72446	1:2000
GABA B1	Abcam; Ab55051	1:2000
GABA B2	Abcam Ab75838	1:2000
Alexa-Fluor 488	JacksonImmuno Research; 711-545-152	1:200
Alexa-Fluor 647	JacksonImmuno Research; 705-605-147	1:200

2.1.5. Statistical analysis

A Shapiro-Wilk test was used to evaluate normality of data distribution. For all data that were found to be normally distributed, a parametric independent t-test was performed. Levene's test was used to determine equality of variance. Independent t-tests were used to evaluate differences between naïve and GSE-incubated conditions. For data that were not normally distributed, a nonparametric Mann Whitney U test was performed to determine differences between naïve and GSE incubated conditions. Statistical analysis was performed using SPSS Statistical Software 28 (IBM, North Castle, NY), and data were considered significant if $p < 0.05$.

3. Results

3.1. Identification of cell types in primary cultures of trigeminal ganglion

Primary rat trigeminal ganglia cultures were used to study the effects of GSE on CGRP secretion and GABAergic-related protein staining. To identify and quantify the cell types present in our cultures, images of differential interference contrast and DAPI stained cells were used to visualize the nucleus, cell body, and processes of all cells. The following four cell types including Schwann cells, satellite glial cells, Aδ fiber neurons, and C fiber neurons were present in the primary cultures (Fig. 1). Aδ and C fiber neurons were differentiated based on cell body size and the round nuclear morphology identified by DAPI staining. Any neuronal cell body larger than 35 μm was considered an Aδ fiber neuron, while any neuronal cell body smaller than 35 μm was considered a C fiber neuron. Schwann cells were identified based on their elongated nucleus and bipolar morphology, while satellite glial cells exhibited a round nucleus and larger cell body with multiple processes. In culture, neurons were present as approximately 14% of the total cell population (339 cells). Aδ neurons comprised 2% (48 cells) and C fiber neurons comprised 12% (291 cells) of total cells. Glial cells were present at approximately 86% (2126 cells) of the total cell population. Satellite glia cells and Schwann cells comprised 59% (1452 cells) and 27% (674 cells) of cell populations, respectively (n = 24).

3.1.1. Characterization of Healthy Origins MegaNatural® BP-Grape Seed Extract

To initially demonstrate that overnight incubation with GSE at 0.5 ng/μL was not causing toxicity, cultured cells were incubated with GSE for 24 h and the change in cell viability was determined. As seen in Table 2, cell viability was not decreased with GSE incubation when compared to untreated naïve control values reported as absorbance at 490 nm. However, cell viability was reduced by incubation with 10% DMSO, which was used as a positive control condition.

3.1.2. Basal CGRP secretion is suppressed by grape seed extract

To determine if GSE could inhibit basal, or unstimulated, secretion of CGRP from trigeminal ganglion neurons, cultures were incubated in HBS containing GSE at a final concentration of 0.5 ng/μL (1.25 ng/well). As seen in Fig. 2, GSE incubation for 60 min caused a significant decrease in

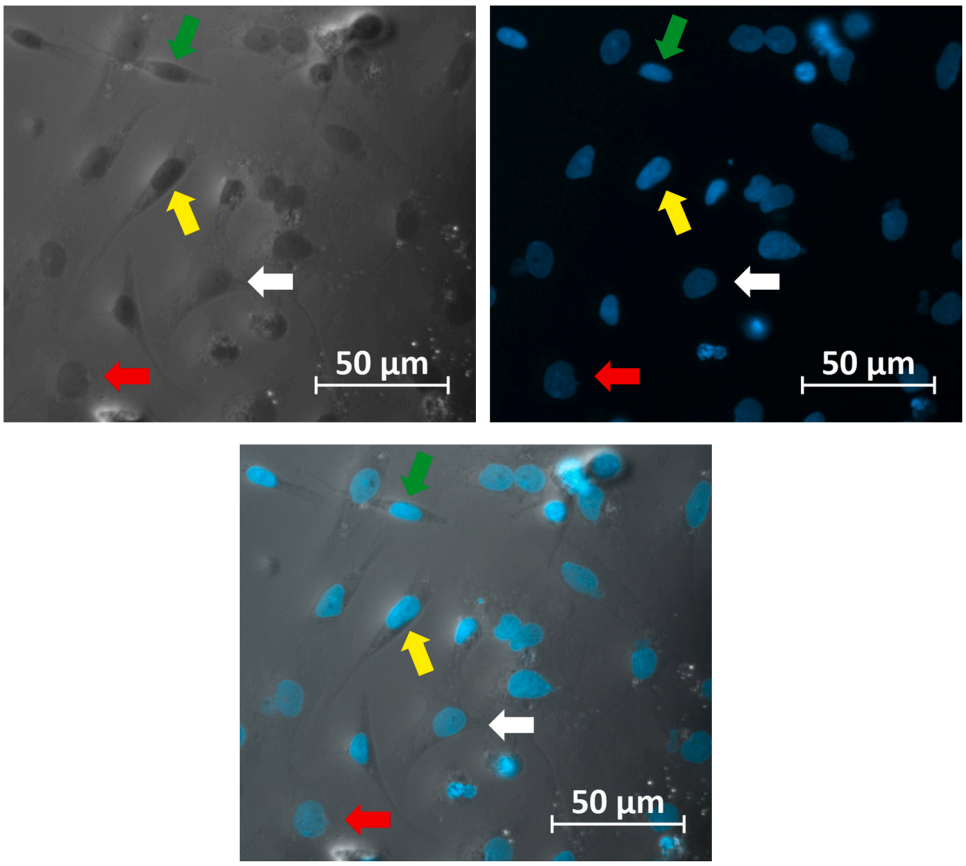


Fig. 1. Characterization of cell types in primary trigeminal ganglia cultures. A 200x representative difference interference contrast (DIC) image (top left), DAPI stained image (top right), and a merged image (bottom panel) are shown. Yellow arrows indicate a satellite glial cell. Green arrows indicate a Schwann cell. White arrows indicate a C fiber neuron. Red arrows indicate an Aδ fiber neuron.

Table 2
Summary of cell viability after overnight GSE incubation.

Sample	Absorbance
Naïve	0.425
GSE 24 h	0.488
10% DMSO 24 h	0.290

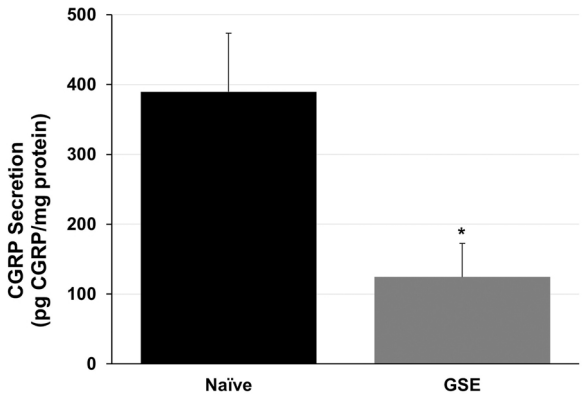


Fig. 2. GSE repression of basal CGRP secretion from trigeminal ganglion neurons. The amount of CGRP secreted into the culture media under unstimulated basal condition and following a 60-minute incubation with GSE was determined by radioimmunoassay. Data are reported as average pg ± SEM of CGRP normalized to total protein level in the well. * = $p < 0.05$ when compared to basal CGRP levels, n = 6 done in duplicate for each condition.

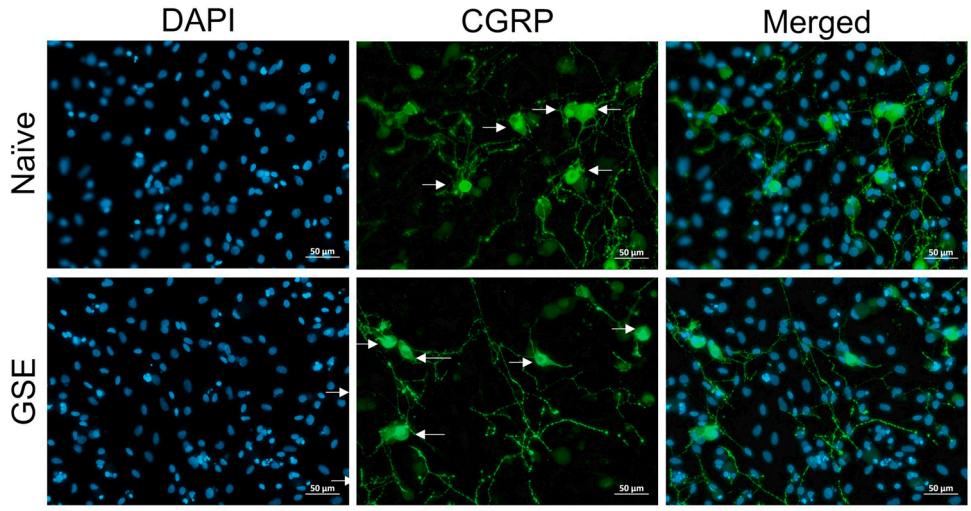
basal CGRP secretion (124.6 ± 48.2 pg, $p = 0.025$, n = 6) when compared to the baseline CGRP level (389.6 ± 83.8 pg). Since the concentration of GSE used for the secretion experiments did not cause a change in cell viability even following overnight incubation (Table 2), it is unlikely that the decrease in CGRP secretion was due to cell toxicity. Taken together, these data provide evidence that the repression of basal CGRP secretion was due to the 60-minute incubation of cultures with GSE.

3.1.3. *Immunostained CGRP levels are not suppressed by grape seed extract*

To determine if GSE was causing a corresponding decrease in the expression of CGRP in neuronal cells, trigeminal cultures were immunostained with antibodies against CGRP and staining intensity measurements compared with naive levels (Fig. 3). In cultured trigeminal ganglia, CGRP was detected only in neurons and their associated neuronal processes. Not all neurons were positive for CGRP expression with less than half of neurons expressing CGRP at higher levels and a small percentage of the population expressing detectable, but low levels of CGRP. The relative intensity of CGRP staining was not altered with overnight GSE incubation (1.05 ± 0.04, $p = 0.459$, n = 6) when compared to naïve conditions (1.00 ± 0.05). Based on a summary of the average relative fold change ± SEM in CGRP staining intensity of GSE incubated compared to naïve condition, GSE did not repress the intracellular level of CGRP.

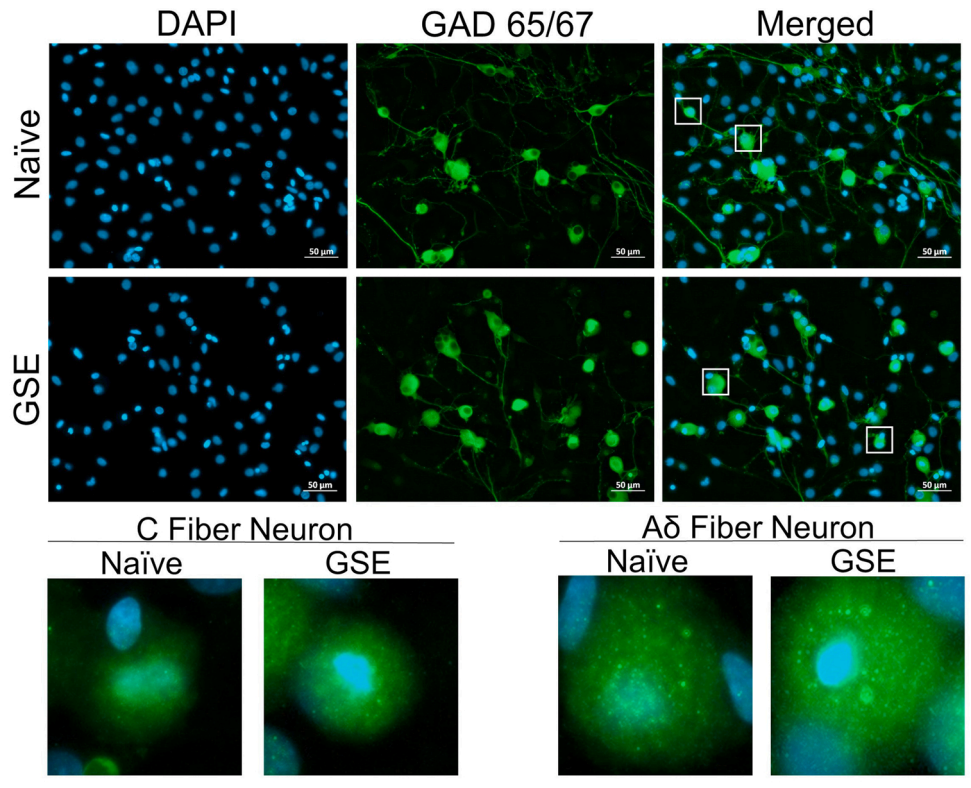
3.1.4. *Grape seed extract stimulates GAD 65/67 expression in neurons*

Immunocytochemistry was utilized to investigate changes in expression of GAD 65 and GAD 67, the enzymes responsible for GABA synthesis, in response to GSE incubation (Fig. 4). In cultured trigeminal ganglia, GAD 65/67 was detected abundantly in both Aδ and C fiber



Condition	Naïve	GSE
Neurons	1.00 ± 0.05	1.05 ± 0.04

Fig. 3. CGRP immunostaining levels in trigeminal neurons are not altered by GSE. All representative images are at 200x magnification. DAPI, CGRP, and merged staining images of naïve (top) and GSE (bottom) conditions. White arrows indicate neuronal cell bodies abundantly expressing CGRP. A summary table of average relative intensity ± SEM is shown. n = 6 done in triplicate for each condition.



Neurons	Naïve	GSE
	1.00 ± 0.06	1.24 ± 0.07*

Fig. 4. GSE increased GAD 65/67 immunostaining levels in trigeminal neurons. DAPI, GAD 65/67, and merged representative images of naïve (top) and GSE (bottom) conditions are shown at 200x magnification. An enlarged image of both neuronal cell types, C fiber and Aδ fiber, and a summary table of average relative intensity ± SEM is shown. * = $p < 0.05$ when compared to naïve levels, n = 9 done in triplicate for each condition.

neuronal cell bodies. A summary of the average relative staining intensity of GAD 65/67 ± SEM compared to naïve levels, whose mean was set equal to one, is shown below the images. The relative intensity of GAD 65/67 staining was significantly increased in neurons with overnight GSE incubation (1.24 ± 0.07, $p = 0.033$, n = 9) compared to naïve conditions (1.00 ± 0.06).

3.1.5. Expression of GABAA is not increased in response to grape seed extract

Changes in expression of the GABAA receptor in response to GSE incubation was investigated using immunocytochemistry (Fig. 5). In cultured trigeminal ganglia, GABAA was detected only in the cell body of Aδ and C fiber neurons and their associated neuronal processes. GABAA staining was not observed in satellite glia or Schwann cells. The relative intensity of GABAA staining was not altered with GSE incubation (0.97 ± 0.11, $p = 0.966$, n = 6) compared to naïve conditions (1.00 ± 0.08). Based on immunostaining intensity measurements, cultures incubated with GSE did not exhibit an increase in GABAA receptor expression in trigeminal neurons.

3.1.6. Neuronal and glial GABAB1 expression is increased by grape seed extract

Immunocytochemistry was used to investigate changes in the expression of the GABAB1 receptor subunit, which is responsible for ligand binding. In cultured trigeminal ganglia, GABAB1 was abundantly expressed in the cell body and processes of Aδ and C fiber neurons (Fig. 6). Low level GABAB1 immunostaining was observed in the cell bodies of satellite glia and Schwann cells. The intensity of GABAB1 staining was significantly increased in both neurons and glia in response to GSE incubation when compared to naïve conditions. Based on immunostaining intensity measurements, GSE incubated cells showed a significant increase in GABAB1 staining intensity in the neurons (1.51 ± 0.09, $p = 0.013$, n = 7) compared to naïve control levels (1.00 ± 0.08). Similarly, a significant increase in GABAB1 staining intensity was observed in the cell body and processes of satellite glial and Schwann cells in response to overnight GSE incubation (1.42 ± 0.05, $p = 0.006$, n = 6) when compared to naïve control levels (1.00 ± 0.10).

3.1.7. Grape seed extract enhances expression of GABAB2 in glial cells

Changes in expression of the GABAB2 receptor, which is responsible for G protein-coupling and functional expression at the cell surface, in response to GSE incubation were investigated using immunocytochemistry. In cultured trigeminal ganglia, GABAB2 was readily detected in neurons and glial cell bodies (Fig. 7). Staining for GABAB2 showed no intensity change with GSE incubation (1.04 ± 0.05, $p = 0.628$, n = 6) in the neurons compared to naïve levels (1.00 ± 0.06). However, the intensity of GABAB2 immunostaining was significantly increased in satellite glia and Schwann cells in response to GSE (1.61 ± 0.08, $p = 0.016$, n = 6) when compared to levels in the naïve condition (1.00 ± 0.07). Based on average relative immunostaining intensity measurements, cells incubated overnight with GSE did not exhibit a significant change in staining intensity in the neurons, in contrast to results seen for GABAB1. However, GSE enhanced GABAB2 expression in satellite glia and Schwann cells, a finding in agreement with the GSE-mediated increase in GABAB1 in these cells.

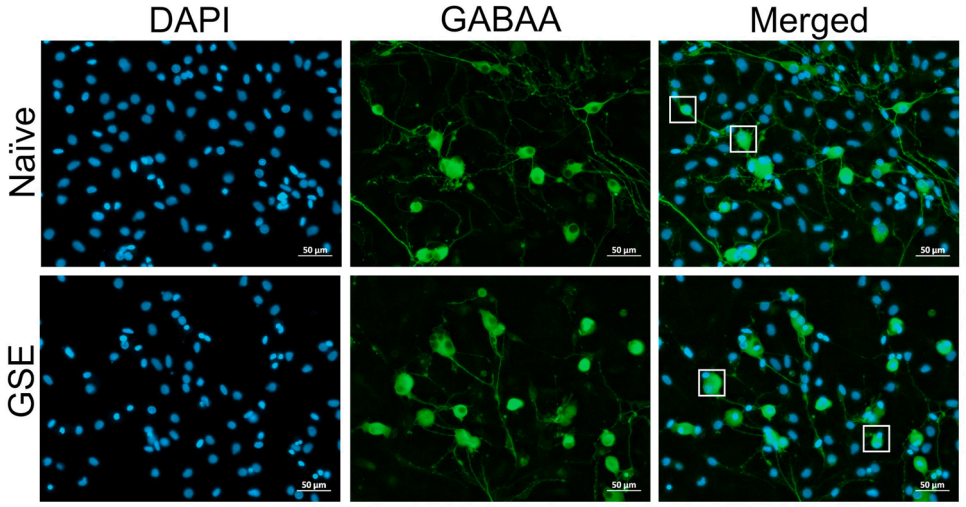
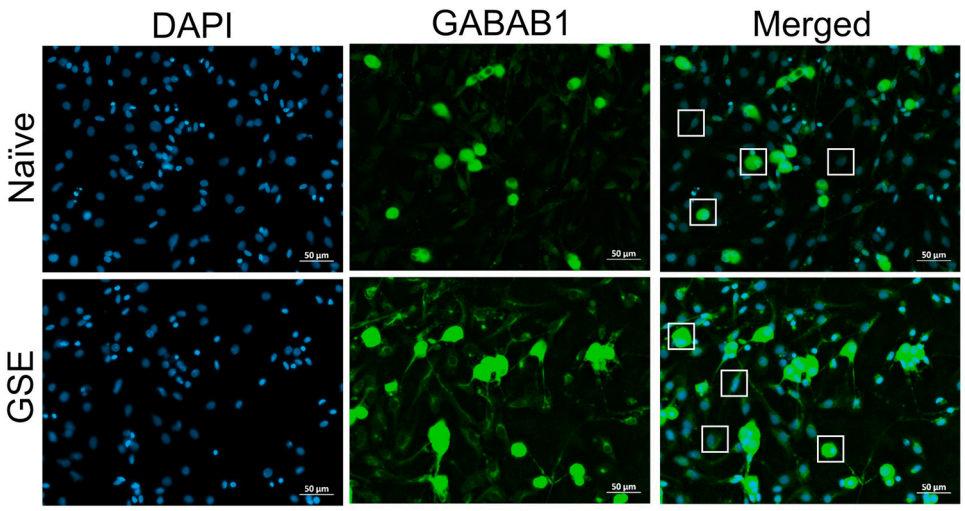
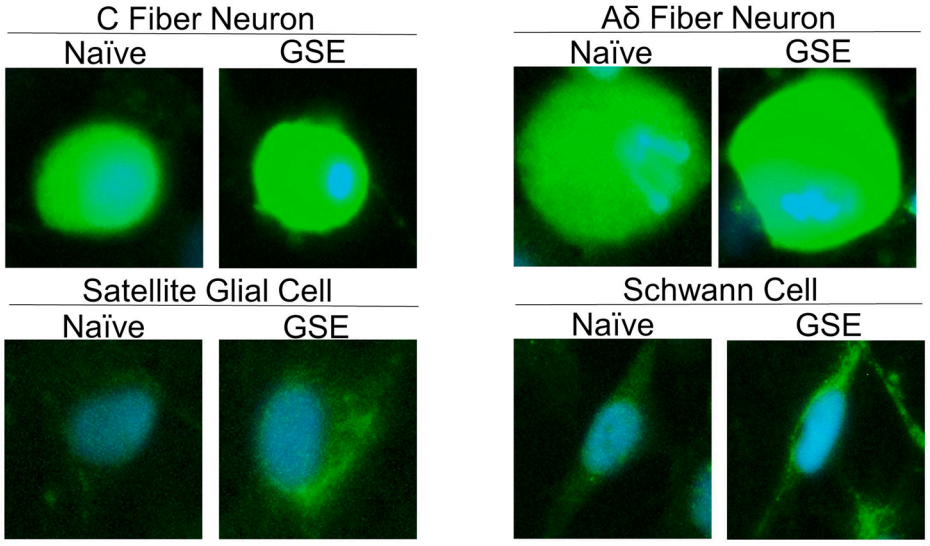


Fig. 5. GABAA immunostaining levels in trigeminal neurons are not altered by GSE. Representative DAPI, GABAA, and merged images of naïve (top) and GSE (bottom) conditions are shown at 200x magnification. An enlarged image for both neuronal cell types, C fiber and Aδ fiber, and a summary table of average relative intensity ± SEM is shown. n = 6 done in triplicate for each condition.



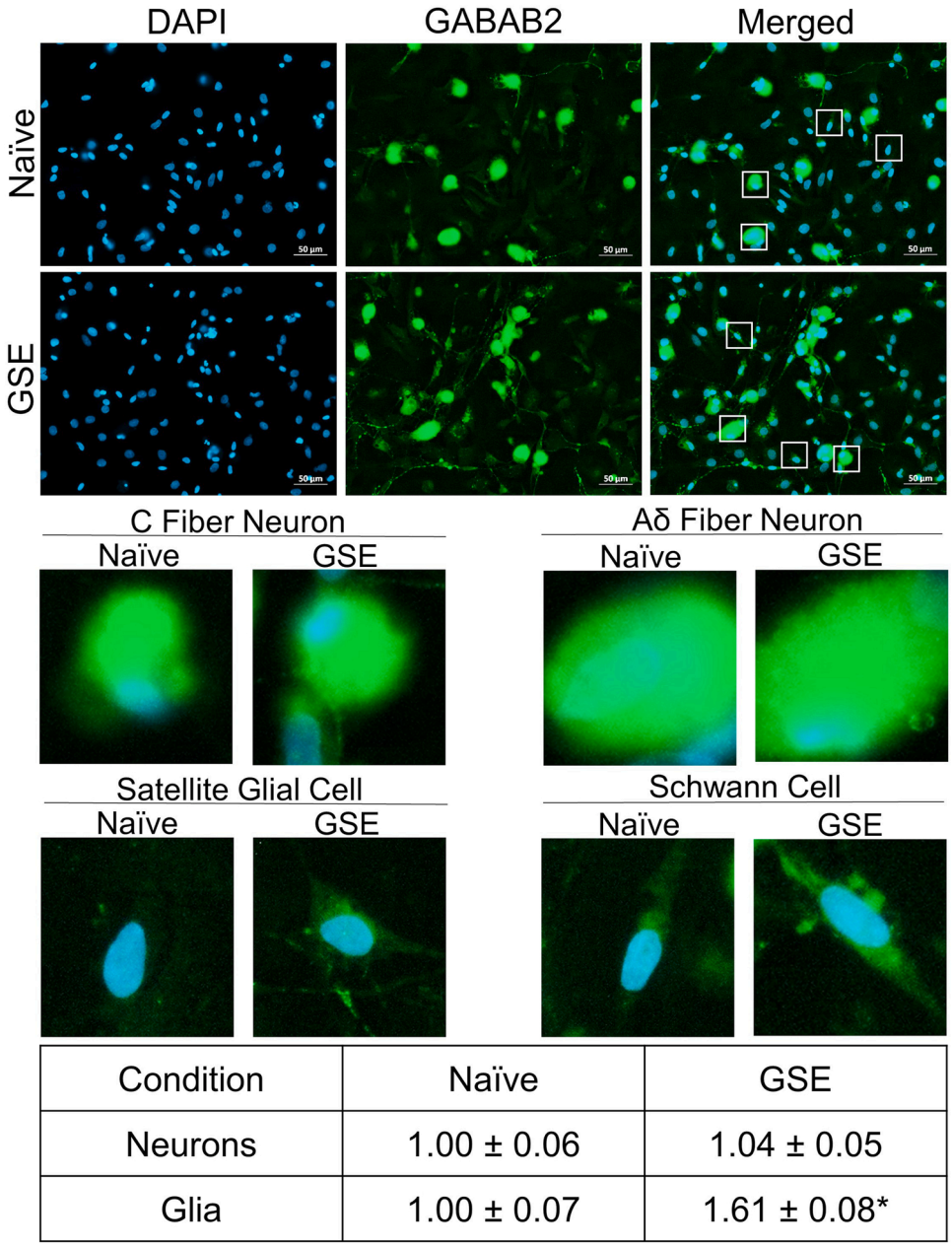


Condition	Naïve	GSE
Neurons	1.00 ± 0.08	1.51 ± 0.09*
Glia	1.00 ± 0.10	1.42 ± 0.05**

Fig. 6. GSE increases GABAB1 immunostaining levels in trigeminal neurons and glia. Representative DAPI, GABAB1, and merged images of naïve (top) and GSE (bottom) conditions are shown at 200x magnification. Enlarged images of neuron and glia are shown for each of the four cell types. A summary table of average relative intensity ± SEM is shown. *=$p < 0.05$ when compared to naïve levels, ** =$p < 0.01$ when compared to naïve levels, n = 7 for neurons and n = 6 for glia done in triplicate for each condition.

4. Discussion

Results from this study demonstrate that incubation of primary trigeminal ganglion cultures with a proanthocyanin-enriched Healthy Origins MegaNatural® BP-Grape Seed Extract solution at a final concentration of 0.5 ng/µL for 60 min significantly inhibited basal CGRP secretion. However, CGRP expression within the neuronal cell body of Aδ and C fiber neurons as assessed by immunocytochemistry remained unaffected even with overnight GSE incubation. This concentration of GSE did not affect cell viability, indicating that the decrease in CGRP secretion was not due to toxicity. The ability of GSE to inhibit constitutive secretion of CGRP from cultured trigeminal ganglion neurons is a novel finding that differs from results from our prior primary trigeminal ganglion studies. For example, incubation of primary cultures with the anti-migraine agents sumatriptan (Durham and Russo, 1999), topiramate (Durham et al., 2006), botulinum toxin type A (Durham and Cady, 2004), and a cocoa extract (Abbey et al., 2008) did not suppress basal CGRP secretion from trigeminal neurons, but only inhibited stimulated CGRP release. Hence, GSE appears to function via a different neuroprotective mechanism to modulate the excitability state of trigeminal neurons by suppressing constitutive CGRP secretion, which involves the fusion of Golgi-derived clathrin-coated vesicles to the plasma membrane (Planells-Cases and Ferrer-Montiel, 2007). GSE inhibition of basal CGRP release from trigeminal ganglion neurons may have important implications since CGRP is known to promote peripheral sensitization of nociceptive neurons via an increase in proinflammatory cytokines and stimulation of nitric oxide release (Durham, 2016; Messlinger et al., 2020). Further, elevated CGRP levels are implicated in TMD and migraine pathology and results from a recent preclinical migraine model provided evidence that CGRP causes cellular changes in the ganglion that mediate allodynia (De Logu et al., 2022). Results from this study are in agreement with our prior finding that dietary inclusion of GSE suppressed basal CGRP expression in the medullary horn of spinal cord tissue (Cady et al., 2010). Thus, we propose that GSE may offer an alternative or adjunctive therapeutic via suppressing CGRP-mediated peripheral and central sensitization of trigeminal



Condition	Naïve	GSE
Neurons	1.00 ± 0.06	1.04 ± 0.05
Glia	1.00 ± 0.07	1.61 ± 0.08*

Fig. 7. GABAB2 expression in trigeminal glial cells is increased by GSE. DAPI, GABAB2, and merged representative images of naïve (top) and GSE (bottom) conditions are shown at 200x magnification. Enlarged images of neurons and glia are shown for each of the four cell types. A summary table of average relative intensity ± SEM is shown. * = $p < 0.05$ when compared to naïve, n = 6 done in triplicate for each condition.

neurons, which is implicated in TMD and migraine pathology.

A major finding from our study was that overnight incubation of trigeminal cultures with GSE caused a significant increase in the neuronal expression of GAD 65 and GAD 67, which are enzymes that mediate production of the inhibitory neurotransmitter GABA (Lee et al., 2019). Under basal, unstimulated conditions, GAD 65/67 immunostaining was readily detected in both Aδ and C fiber neurons and hence, these cells would function as the primary source of endogenous GABA production and release in the primary cultures. While GAD 67 is the isoform responsible for basal or constitutive synthesis of GABA in the cytoplasm of the cell body, GAD 65 is a plasma membrane anchored protein that synthesizes GABA for loading into synaptic vesicles and its release under stimulatory conditions (Buddhala et al., 2009). The secretion of GABA from trigeminal neurons would be expected to exert an inhibitory effect on the excitability state of trigeminal ganglion neurons and glial cells expressing GABA receptors. Our finding that only trigeminal neurons express GAD 65 and GAD 67 under basal conditions is in agreement with prior studies that reported that trigeminal neurons, but not glial cells, express GAD 65 and GAD 67 mRNA (Hayasaki et al., 2006) and that GAD 65 and GAD 67 are colocalized with CGRP (Okuno et al., 1994). To our knowledge, this is the first evidence of a polyphenolic-enriched extract promoting increased expression of GABA producing enzymes in neuronal cells.

GSE also stimulated neuronal expression of the GABAB1 receptor subunit, which is responsible for binding GABA and forming a functional complex with the GABAB2 subunit (Evenseth et al., 2020). The GABAB1 receptor subunit was significantly upregulated in trigeminal ganglion Aδ and C fiber neurons incubated overnight with GSE. However, the level of expression of the GABAB2 receptor subunit, which couples to the inhibitory G protein, remained unchanged in both types of neurons in response to GSE. The synthesis and release of CGRP from trigeminal neurons is enhanced by the stimulatory G protein-PKA-cAMP coupled

pathway, and this pathway is implicated in the development of peripheral sensitization (Cady et al., 2011; Cornelison et al., 2016). Thus, the GSE-mediated increase in the functional expression of the GABAB receptor via upregulation of GABAB1 in trigeminal neurons could directly oppose the cellular effects of stimulatory agents such as nitric oxide and cytokines that facilitate CGRP release (Bowen et al., 2006; Durham et al., 2006). This change in neuronal receptor expression would allow for more GABA binding, which could partially explain some of the inhibitory effects of GSE observed in preclinical orofacial pain models (Cady et al., 2010; Cornelison et al., 2020, 2021; Woodman et al., 2022). Increased activation of the GABAB receptors is known to promote potassium efflux, inhibition of calcium influx, and a decrease in cyclic AMP levels in the neurons, resulting in a lower neuronal excitability state (Padgett and Slesinger, 2010; Evenseth et al., 2020). Through these inhibitory G protein-coupled mechanisms, activation of the GABAB receptors would lead to a lower excitability state of the neuron and hence, a greater inflammatory stimulus would be required to cause depolarization of Aδ and C fiber neurons. Furthermore, we propose these GSE-mediated cellular events on the GABAergic system may be involved in inhibiting the release of neurotransmitters and neuropeptides such as CGRP from primary trigeminal neurons. However, future pharmacological studies will be needed to provide direct evidence of the role of the GABAergic system since it is plausible that the GSE-mediated rapid inhibition of CGRP release may involve a different mechanism.

In contrast to GSE upregulation of GABAB receptors in trigeminal neurons, overnight incubation with GSE did not cause a significant change in GABAA expression as assessed by immunocytochemistry. This finding provides evidence that the inhibitory effects of GSE are being mediated primarily via upregulation of the GAD 65 and GAD 67 enzymes, and the GABAB1 receptor subunit in trigeminal neurons. Binding of GABA to the GABAA receptor, which functions to inhibit neuronal activation, couples to the movement of negatively charged chloride ions across the plasma membrane to cause hypopolarization of neurons (Mihic and Harris, 1997; Barker and Hines, 2020). Interestingly, activation of the GABAA receptor, but not GABAB, was shown to be associated with the anti-nociceptive effect of non-invasive vagus nerve stimulation (nVNS) in a preclinical chronic migraine model (Cornelison et al., 2020). Thus, it appears that the inhibitory effect of GSE and nVNS in the trigeminal system function via activation of different GABA receptors. These findings support the notion that there may be an enhanced therapeutic benefit of using them together in the management of migraine and TMD. Hence, GSE appears to be exerting its beneficial effects only through the GAD 65 and GAD 67 enzymes and the metabotropic G protein-coupled GABAB receptor of trigeminal neurons. While neurons are a critical cell type involved in inflammation and nociception, the central role of glial cells in modulating the excitability state of neurons and the development of peripheral sensitization is well established (Messlinger et al., 2020).

In addition to modulating trigeminal neurons, overnight incubation with GSE induced a significant increase in the expression of the GABAB1 and GABAB2 receptor subunits in the cell body and processes of satellite glia in our primary trigeminal ganglion cultures. Our finding agrees with prior reports that GABAB, but not the GABAA, receptors are expressed in glia (Vit et al., 2009; Takeda et al., 2013, 2015). Given the important role of glial cells in modulating the excitability state of primary sensory neurons (Takeda et al. 2007; Freeman et al. 2008; Ohara et al., 2008), this finding may offer a novel strategy for targeting peripheral glial cells to suppress or possibly even reverse development of peripheral sensitization. Prolonged sensitization of trigeminal neurons is known to involve CGRP and enhanced neuron-glial cell communication via paracrine signaling and the formation of gap junctions (Durham, 2016). For example, prior studies from our lab have shown that CGRP stimulates nitric oxide synthesis and release from trigeminal ganglion glial cells and promotes release of many proinflammatory cytokines, such as TNF-α, that stimulate neurons to release even more CGRP (Li et al., 2008; Vause and Durham, 2010). Thus, inhibiting CGRP-mediated cellular events in glial cells would suppress the development and maintenance of the neuron-glia inflammatory loop within the ganglion. Increasing the GABAergic response in satellite glial cells would be expected to suppress peripheral sensitization and activation of trigeminal neurons and the formation of gap junctions between the neuronal cell body and satellite glial cells, which is implicated in the transition from acute to chronic pain (Garrett and Durham, 2009; Durham and Garrett, 2010). Similarly, GSE promoted upregulation of GABAB1 and GABAB2 receptor subunits in the cell body and processes of Schwann cells, which were recently implicated in the underlying pathology of migraine (De Logu et al., 2022). In that study, CGRP binding to its receptor on Schwann cells led to the production of nitric oxide and modulation of ligand-gated ion channels on Aδ and C fiber neurons to cause cellular changes associated with allodynic pain signaling. Thus, upregulation of the GABAB receptor subunits in Schwann cells would inhibit the synthesis and release of nitric oxide, which is stimulated by CGRP. Our results provide evidence of a neuroprotective role of GSE via modulation of trigeminal ganglion glial cells implicated in peripheral sensitization and pain signaling.

Findings from this study extend our understanding of how the nutraceutical GSE functions to suppress trigeminal sensitization and activation by enhancement of GABAergic signaling in both neurons and glial cells in the trigeminal ganglion (Fig. 8). Previously, we demonstrated that dietary inclusion of GSE functions to enhance descending inhibitory pain signaling in preclinical models of migraine and TMD via activation of serotonergic, GABAergic, and endocannabinoid receptors to suppress central sensitization (Cornelison et al., 2021; Woodman et al., 2022). Further, the cellular effects of GSE were shown to involve upregulation of basal levels of the enzyme MKP-1, which inactivates the pro-inflammatory MAP kinases, and the glutamate transport proteins GLAST and GLT-1 to lower the extracellular concentrations of this stimulatory neurotransmitter (Cady, Hirst et al. 2010). In summary, we propose that GSE functions in a neuroprotective role to suppress peripheral sensitization of trigeminal neurons via inhibition of CGRP secretion, promotion of neuronal expression of GAD 65 and GAD 67, and enhanced GABAB receptor expression in neurons and glia. Given that elevated levels of CGRP are implicated in TMD and migraine pathology, GSE may be beneficial as a complementary or adjunctive approach for the clinical management of migraine and TMD and possibly other types of orofacial pain.

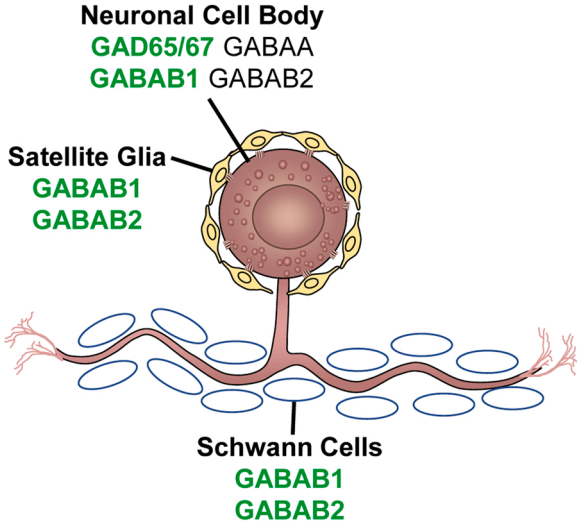


Fig. 8. Summary of GSE modulation of GABAergic system in trigeminal ganglion. Proteins upregulated by GSE in this study are highlighted by green font.

Ethical statement

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

CRediT authorship contribution statement

Sophia R. Antonopoulos was responsible for generation of the data, performing analysis, and writing and editing of the manuscript. Paul L. Durham was responsible for study design, interpretation of results, and writing and final editing of manuscript.

Acknowledgments

We would like to thank Sara Woodman and Chloe Keyes for their technical assistance and Angela Goenrdt for her assistance with animal maintenance. We would also like to thank the National Institutes of Health (NIDCR DE024629) for providing funding for this study.

Conflicts of Interest

The authors declare no competing financial interests.

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