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**INVESTIGATION OF THE NEUROPROTECTIVE EFFECTS OF GRAPE SEED
EXTRACT ON TRIGEMINAL GANGLION PRIMARY CULTURES**

A Master's Thesis

Presented to

The Graduate College of

Missouri State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science, Biology

By

Sophia Rose Antonopoulos

August 2022

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INVESTIGATION OF THE NEUROPROTECTIVE EFFECTS OF GRAPE SEED EXTRACT ON TRIGEMINAL GANGLION PRIMARY CULTURES

Biology

Missouri State University, August 2022

Master of Science

Sophia Rose Antonopoulos

ABSTRACT

Migraine and temporomandibular disorders (TMD) are prevalent, debilitating orofacial pain conditions involving peripheral and central sensitization of the trigeminal system. The pro-inflammatory neuropeptide calcitonin gene-related peptide (CGRP), which is synthesized and secreted from trigeminal ganglion neurons, is implicated in the underlying pathology of migraine and TMD. Secreted CGRP modulates the excitability state of neurons and glial cells that express CGRP receptors. Recent studies from our lab in preclinical models of migraine and TMD have provided evidence that dietary supplementation with a proanthocyanin-enriched grape seed extract (GSE) inhibits trigeminal pain signaling. The effect of GSE was blocked by an antagonist of the GABAB receptor, which is expressed on primary trigeminal neurons. My study aimed to investigate the cellular mechanisms by which GSE functions to modulate CGRP expression using primary trigeminal ganglion cultures. The effect of GSE on CGRP secretion from trigeminal neurons was determined by radioimmunoassay. To determine if the effects of GSE involve modulation of CGRP expression or the GABAergic system, changes in the expression of CGRP, GAD 65/67, GABAA receptor, and GABAB1 and GABAB2 receptor subunits were investigated by immunocytochemistry. GSE significantly inhibited the basal level of CGRP secretion but did not alter the neuronal expression of CGRP. GAD 65/67 expression levels in neurons were increased in response to GSE incubation. No change in the neuronal expression of GABAA was observed in response to GSE. GABAB1 expression in neurons, satellite glial cells, and Schwann cells increased in response to GSE. GABAB2 expression was elevated in satellite glia and Schwann cells. My findings support the notion that GSE inhibition of basal CGRP secretion involves increased neuronal GAD 65/67 and GABAB receptor expression. GSE repression of CGRP release in the ganglion coupled with increased GABAB1 and GABAB2 glial cell expression would suppress neuronal excitability and the development of peripheral sensitization. In summary, I propose that GSE mediates neuroprotective effects that support its potential as a nutraceutical therapeutic in the management of migraine and TMD.

KEYWORDS: neuroprotective, trigeminal ganglion, peripheral sensitization, calcitonin gene-related peptide, grape seed extract, gamma-aminobutyric acid

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August 2022

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In the interest of academic freedom and the principle of free speech, approval of this thesis indicates the format is acceptable and meets the academic criteria for the discipline as determined by the faculty that constitute the thesis committee. The content and views expressed in this thesis are those of the student-scholar and are not endorsed by Missouri State University, its Graduate College, or its employees.

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I dedicate this thesis to my parents, Tamara and Constantine Antonopoulos.

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INTRODUCTION

Migraine and Temporomandibular Disorder

Migraine is a severe form of headache that causes intense orofacial pain and is the most common neurological disease [14; 44]. Migraine attacks are characterized by an intense, debilitating, throbbing pain in the head and face and a strong aversion to light (photophobia), sounds (phonophobia), and odors. In almost a third of migraineurs, attacks can be accompanied by an aura, which is usually some form of visual disturbance, as well as severe nausea that can cause vomiting [50; 61]. The pain, which can last from 4 to 72 hours, is typically experienced on one side of the person's head but can be bilateral. Migraines occur in stages; it first begins with a prodrome phase. This phase may consist of digestive issues, mood changes, fatigue, and neck and shoulder stiffness [94]. Hours after the prodrome phase, the migraine attack happens. This is the stage when the head pain and nausea are most pronounced. Once the pain and nausea begin to wear off, the postdrome phase begins. In this stage, which can last for more than 24 hours, the person is typically very tired and may be confused. This condition is referred to as brain fog. When people experience a migraine, they are not able to go to work or carry out normal daily functions for at least a couple of days. This greatly impacts the quality of life of migraineurs. Migraine is classified either as episodic or chronic based on the number of headache days experienced each month with chronic migraineurs reporting more than 15 headache days per month. There are currently numerous pharmacological and alternative therapeutic strategies to help prevent and abort migraine attacks [13]. Despite the recent progress, many migraine sufferers report inadequate pain and symptom relief along with the

experience of negative side effects. Hence, there remains a need to better understand migraine pathology and develop safe and effective therapeutic strategies.

Based on patient surveys, a variety of environmental and internal triggers have been identified that can initiate a migraine attack [9; 42; 50; 86]. Hormonal changes in women, stress, excess caffeine, poor sleep quality, and a change in weather can all be triggers. Migraine is estimated to affect roughly 18 percent of the global population. Women are 2-3 times more likely than men to experience migraines. This sex difference is partly attributed to the hormonal differences between the two sexes that promote underlying cellular and molecular differences. Another risk factor for experiencing migraines is family history or one's genetic background; having a family member with migraines increases your likelihood of experiencing a migraine during your lifetime [61]. Migraine is often comorbid with another prevalent orofacial pain condition known as temporomandibular disorder or TMD [25; 40].

TMD is a group of medical conditions involving dysfunction of the temporomandibular joint (TMJ) and/or associated muscles of mastication [89]. The TMJ is the only dually hinged joint in the human body, and TMD pathology can involve inflammation and pain in one or both joints. Following injury, the joint capsule can become inflamed. This leads to swelling, redness, and tenderness near the joint. Depending on the severity of the injury and the body's healing response, an injury can lead to development of chronic pain in the facial region surrounding the jaw and near the ear. TMD can make chewing food more difficult, more painful, and inhibit proper jaw movement during normal activities such as speaking [71]. When a patient goes in for diagnosis, the exact cause of TMD development is usually never determined. Injury to the jaw, genetic predisposition, and teeth clenching and grinding caused by an unmanaged stress response can contribute to the pathology associated with TMD [67; 88]. Treatment regimens for TMD

include pain relief with over-the-counter anti-inflammatory medications, lifestyle modification to avoid any triggers that aggravate the jaw, and surgery as a last resort [73]. Surgeries performed to repair a damaged TMJ capsule do not always help the patient, and oftentimes can pose a risk for causing further damage and long-term pain and disability. The prevalence of TMD is reported as 5-12% in the U.S. population. Like migraine, TMD is two times more common in women than in men [8; 67]. Both migraine and TMD are prevalent orofacial pain diseases characterized by sensitization and activation of the primary afferent nerves within the trigeminal ganglion [20; 80].

Trigeminal Ganglion

The trigeminal ganglion is the part of the peripheral nervous system responsible for connecting the head and facial region to the central nervous system [46]. This collection of nerve cells allows for transmission of sensory information, such as chemical, mechanical, and thermal signals from peripheral tissues to the spinal cord and brain [83]. Humans have two trigeminal ganglia, with one on each side of the body. They lie in a depression that is located next to the temple, in front of the ears, and behind the eyes. The trigeminal ganglion has three main nerve branches including the ophthalmic (V1), maxillary (V2), and mandibular (V3) [82; 84; 85]. Each branch conveys sensory signals from a different area of the face to the upper spinal cord. The V1 branch provides sensory innervation to the upper facial region and the top of the frontal area of the head. V2 provides innervation of the sinus/nasal region, and V3 innervates the jaw and lower facial region. V3 is considered a mixed nerve since it also provides motor function for mastication.

The trigeminal ganglion consists of primary afferent neurons, or neurons that convey signals from the peripheral nervous system to the central nervous system [20; 79; 80]. This includes A δ fiber and C fiber neurons (Fig. 1). The cell body and processes of A δ fiber neurons are larger than those of C fiber neurons. The diameter of the cell body of A δ fiber neurons ranges between 35 and 50 μm , while the cell body diameter of C fiber neurons is typically between 20 and 30 μm . A δ fibers transmit the sensation of temperature as well as sharp or acute pain. Since all A δ fibers are myelinated, they are responsible for very fast neurotransmission that functions to protect peripheral tissues from injury or damage. A δ fiber neurons respond to signals by releasing the excitatory neurotransmitter glutamate and other excitatory neurotransmitters into the spinal cord to cause sensitization and activation of secondary neurons. The secondary neurons carry the signal to the thalamus. Tertiary neurons will then transmit the signal to the sensory cortex for higher level processing and initiating a proper behavioral response. Neurotransmitters and neuropeptides released from A δ fiber neurons can also cause sensitization and activation of glial cells such as astrocytes, microglia, and oligodendrocytes [47; 51]. These glial cells function to modulate the signaling pathway within the central nervous system.

The other major cell type is the C fiber neurons. C fiber neurons transmit pain, temperature, and the sensation of itch from peripheral tissues to the spinal cord [83]. In contrast to A δ fibers, C fibers are unmyelinated. Therefore, C fibers transmit sensory information slowly and are involved in the more sustained transmission of sensations such as chronic or burning pain. C fiber neurons transmit pain signals via the release of neurotransmitters and neuropeptides like substance P and calcitonin gene-related peptide (CGRP) to secondary neurons [52]. These neuropeptides can also promote sensitization and activation of glial cells such as

satellite glia and Schwann cells within the ganglion and astrocytes, microglia, and oligodendrocytes in the spinal cord [28]. Sensitization and activation of A δ and C fiber neurons within the three branches of the trigeminal ganglion are implicated in prevalent orofacial pain conditions including migraine and TMD [20]. Activation of these primary sensory neurons leads to increased neuron-glia signaling and the subsequent development and maintenance of peripheral sensitization within the trigeminal ganglion and central sensitization in the spinal cord [6; 22; 52].

Peripheral sensitization of primary sensory neurons, which is implicated in migraine and TMD pathology, is associated with a lower activation threshold to cause depolarization [28]. A lower activation threshold means that less stimuli are required to cause activation of the neurons. Development of peripheral sensitization is known to involve enhanced neuron-glia cell communication via paracrine signaling and gap junctions to mediate an inflammatory response [31; 46; 90]. In addition to the primary afferent neurons, two types of glial cells are present in the trigeminal ganglion including satellite glial cells and Schwann cells (Fig. 1). The cell body of glial cells are smaller than those of neurons. Their nuclei have an elongated shape, while neuronal nuclei have a very spherical and round morphology. Satellite glial cells are more abundant than Schwann cells in the ganglion. Satellite glial cells form a continuous layer wrapping around the neuronal cell body that functions to provide nutrients and to modulate the excitability state of the neurons [28]. A key protein expressed by satellite glial cells is the ion channel protein Kir 4.1, which functions as an inwardly rectifying potassium channel to remove extracellular potassium ions, and hence decrease neuronal excitability [98]. Together, the neuronal cell body and satellite glial cells form what is referred to as a functional unit that develops within the first few weeks after birth in rats [30]. These cells are capable of forming

gap junctions, a type of communication where two cells connect their cytoplasm to share small molecules such as glucose, amino acids, and ions [43]. The gap junctions allow for enhanced direct communication by metabolically and electrically coupling neurons and satellite glial cells during migraine and TMD pathology. Both types of glia, satellite glia and Schwann cells, function as neuromodulators to regulate the excitability state of trigeminal neurons [62].

Within the ganglion, Schwann cells have two distinct populations, myelinating, and non-myelinating Schwann cells [45; 62]. Myelin, which forms a sheath around nerve axons to allow for fast electrical conductance, is essential for axons to be able to transmit signals 100 times more quickly than unmyelinated fibers. Myelinating Schwann cells wrap around the axon of all A δ nerve fibers. They respond to physiological cues of the axon to determine the thickness and length of the myelin sheath produced. Non-myelinating Schwann cells wrap around multiple C nerve fibers, forming a Remak bundle. Non-myelinating Schwann cells are thought to play a role in maintenance and nutrient delivery to the axon of trigeminal C fiber neurons.

Calcitonin Gene-Related Peptide

The most studied neuropeptide in the trigeminal ganglion is calcitonin gene-related peptide or CGRP and is the primary target of the most effective anti-migraine drugs [27; 35; 66]. This 37 amino acid proinflammatory neuropeptide is abundant in both the peripheral and central nervous systems [75; 76]. There are two different isoforms of CGRP, α -CGRP and β -CGRP [74]. α -CGRP is the form expressed in A δ and C fiber nociceptive neurons within the trigeminal ganglion. β -CGRP is expressed in enteric neurons that regulate multiple cellular functions within the gut. α -CGRP is the focus of my thesis project and will be referred to as CGRP in the following text. The cellular effects of CGRP are mediated via activation of the CGRP receptor

[7; 52]. The CGRP receptor is comprised of two main transmembrane proteins, receptor activity-modifying protein (RAMP1) and calcitonin like-receptor (CLR). There are 3 RAMP family proteins, but RAMP1 is primarily involved in CGRP signaling since RAMP1 has the highest affinity for CGRP. A primary function of RAMP1 is to facilitate the movement of the receptor complex to the surface of the cell, which controls when the CGRP ligand can bind and allows for specificity. The functional CGRP receptor is formed when RAMP1 and CLR form a complex. In addition to the two main proteins, the CGRP receptor is associated with two proteins within the cytoplasm. These are the receptor coupling protein (RCP) and the α subunit of the Gs protein, which is the CLR receptor subunit. Gs proteins are stimulatory guanine nucleotide-binding proteins that act as a switch to initiate intracellular cell signaling. The α subunit of the Gs protein is responsible for the transduction of the signal for the CGRP pathway. The RCP protein is necessary for the Gs α subunit to properly transmit the signal. Once the Gs α protein is activated, it binds and stimulates the enzyme adenylate cyclase [17]. Activation of adenylate cyclase leads to a large increase in the intracellular level of the secondary messenger cyclic AMP (cAMP) via a structural change of ATP. cAMP then activates the multifunctional enzyme protein kinase A (PKA). PKA stimulates synthesis and release of nitric oxide, cytokines, and other proinflammatory molecules from neurons and glial cells. The CGRP receptor is expressed on neurons, satellite glial cells, and Schwann cells within the trigeminal ganglion and secondary neurons, astrocytes, and microglia in the spinal cord [26; 52].

At normal physiological levels, CGRP regulates many cellular functions that may be protective. However, elevated levels of CGRP are associated with migraine and TMD pathology [7; 17]. Following the discovery of CGRP, it was quickly identified as the most potent vasodilatory neuropeptide. CGRP is synthesized in the neuronal cell body where it is stored in

dense-core vesicles in the cytosol and neuronal processes. CGRP secretion is stimulated by activation of the neurons within the trigeminal ganglion. When released perivascularly, it promotes neurogenic inflammation, which is characterized by blood vessel dilation, plasma protein extravasation, degranulation of mast cells, and recruitment of immune cells [5]. This type of inflammatory response can serve a protective function by preventing ischemic events, minimizing tissue damage, promoting tissue repair, and restoring homeostasis. CGRP also functions as a pain signaling molecule to mediate sensitization and activation of primary neurons, secondary neurons, and glial cells [28]. When sensitized, the neurons in the peripheral nervous system have a higher excitability state, which correlates to a lower activation threshold. Thus, a smaller change in membrane potential is required to initiate a cellular response and facilitate pain signaling. The neuronal membrane potential is referring to the difference in electrical charge within the cell compared to the environment outside of the cell. The cytoplasm of neurons at rest is negatively charged to a potential of about -60 to -75 millivolts. A higher excitability state would mean that the potential of the cell is still negative, but to a lesser degree. Hence, it is easier for a stimulus to open a ligand-gated positive ion channel and cause depolarization of the cell, and subsequent release of CGRP and other neurotransmitters.

CGRP not only initiates peripheral sensitization, but it perpetuates this hyperexcitable state by creating an inflammatory feedback loop [17; 97]. CGRP acts as an autocrine signal to stimulate the synthesis and secretion of more CGRP by binding to the CGRP receptor on the trigeminal neuron that originally released it. It also acts as a paracrine signal to stimulate proinflammatory cytokine release, such as tumor necrosis factor- α (TNF- α), from satellite glial cells. Nearby release of TNF- α stimulates further CGRP transcription and secretion [10; 36]. Additionally, CGRP stimulates synthesis of nitric oxide release from neurons as well as both

Schwann and satellite glial cells [58]. Nitric oxide is another potent vasodilator and functions in collaboration with CGRP to prolong the inflammatory response in response to trigeminal nerve activation [4]. The increase in neuron-glial cell communication involving CGRP, cytokines, and nitric oxide mediates peripheral sensitization and is implicated in chronic orofacial pain conditions [28].

In the case of migraine, a chronic orofacial pain disease, release of CGRP from the trigeminal ganglion results in inflammation in the meninges of the brain [44]. This inflammation is what causes the severe pain associated with a migraine attack. Given that elevated CGRP levels in cerebral spinal fluid, saliva, and serum have been reported during a migraine attack, it is not surprising that this neuropeptide has become a key target of migraine therapeutics including drugs such as the triptans and gepants, onabotulinumtoxinA, and biologics such as CGRP monoclonal antibodies that are directed against the peptide or its receptor [72]. Inhibition of CGRP release and prevention of CGRP receptor activation in neurons and glia in the trigeminal ganglion and spinal cord has helped migraine patients manage their pain and decrease associated symptoms. Based on published work from our laboratory, other therapeutic strategies such as inclusion of dietary supplements that function as nutraceuticals have been shown to be effective in inhibiting pain signaling and inflammation and inhibit CGRP expression in preclinical models of migraine and TMD [1; 15; 16; 18; 21; 24; 48; 101].

Grape Seed Extract

Grapes, grape leaves, sap, and grape seeds have been used in ancient medicine for a variety of health benefits [59; 65]. The grape seed extract (GSE, Healthy Origins MegaNatural® BP-Grape Seed Extract) used in our prior published studies [18; 21; 24; 101] and my thesis

research is a commercially available extract made from both red and white California grape varieties. This extract is enriched in polyphenols. Polyphenols are a large, diverse class of secondary plant compounds known to protect against inflammation and oxidative stress [2]. A major source of the grape's polyphenolic content is found in the seeds. One of the main classes of polyphenols found in GSE is proanthocyanins. These polyphenols are powerful antioxidants and as such provide a broad range of benefits by reducing oxidative stress. Previous findings from our laboratory have provided evidence that the addition of GSE as a daily supplement to the drinking water of Sprague-Dawley rats inhibits 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 [18; 21; 24; 101]. Importantly, the inhibitory effect of GSE involves repression of basal CGRP expression in the spinal cord. Elevated levels of CGRP in the spinal cord are known to promote both central and peripheral sensitization of trigeminal nociceptive neurons [54]. In summary, there are multiple lines of evidence to support the notion that GSE functions in a neuroprotective capacity via modulation of neuronal CGRP expression. This nutraceutical offers a non-pharmacological therapeutic modality for migraine and TMD and possibly other chronic inflammatory pain conditions.

Supplements, like GSE, are considered a nutraceutical rather than a pharmaceutical. Nutraceuticals are natural bioactive compounds that show physiological benefit to human health. They can be used not only as treatment for a disease, but as a preventative before pathology begins. In a society where our approach to human health is primarily focused on treatment after pathology has occurred, identification of modalities that prevent the development and progression of disease remains a much-needed area of emphasis. Pharmaceuticals typically are advantageous in treating a specific disease by inhibiting the molecule that is implicated in the

underlying pathology, such as CGRP in migraine. In contrast, nutraceuticals enhance the expression of proteins and pathways that suppress inflammation and pain signaling, and function to restore and maintain healthy physiological systems. Another major difference is the fact that recommended therapeutic doses of pharmaceuticals can be associated with negative side effects. Nutraceuticals usually do not cause any significant side effects. They are consumed at lower levels in one's diet on a regular basis and function not as abortive therapies, but as preventative therapeutics. Combining nutraceuticals with pharmaceuticals could offer an additive or synergistic therapeutic benefit and hence function as adjunctive therapies. Taking a pharmaceutical in addition to a nutraceutical may allow the pharmaceutical to be taken at a lower dose, perhaps decreasing the burden of side effects, and increasing its efficacy.

Based on our findings and supported by the National Institutes of Health Complementary and Integrative Health website, GSE should be considered in the management of pain associated with migraine, TMD, and other chronic orofacial pain conditions. In support of this notion, dietary supplementation with GSE was found to inhibit nociception and latent sensitization in preclinical models of chronic TMD and episodic migraine [18; 21; 24; 101]. Our lab has previously documented how GSE functions to prevent central sensitization, but there is a gap in knowledge about how GSE is functioning in the peripheral system at the level of the trigeminal ganglion. Polyphenols can cross the blood brain barrier [39], making them capable of exerting effects in both the central nervous system and the peripheral nervous system. In the central nervous system, polyphenol-enriched GSE effects involve activation of the serotonin receptor 5-HT, the endocannabinoid receptors CB1 and CB2, and the GABAB receptor to modulate descending pain [21; 24; 101]. Other widely used pharmaceutical pain relievers, such as morphine and opioids, also work through enhancing the inhibitory descending pain modulation

pathway to prevent pain signaling. Elucidating the cellular mechanisms of how GSE targets the peripheral nervous system in the trigeminal ganglion to modulate descending pain pathways mediated by CGRP was the focus of my thesis.

GABAergic System

Results from our preclinical studies have demonstrated that the inhibitory properties of GSE involve in part, activation of gamma-aminobutyric acid (GABA) receptors [21] within the spinal cord. GABA is a key inhibitory neurotransmitter in the brain and spinal cord of mammals [38]. Lack of GABA in the nervous system can lead to psychological diseases such as anxiety and depression[37; 77]; these conditions are often comorbid with CGRP-implicated orofacial pain diseases like TMD and migraine[64; 87]. Although glutamate is the structural precursor of GABA, they exert opposing effects on nerve cells. Glutamate is the most abundant excitatory neurotransmitter in the brain and spinal cord, while GABA is the most abundant inhibitory neurotransmitter. Hence, while glutamate facilitates neuronal activation and pain signal transduction, GABA inhibits neurons from being able to relay inflammatory and painful information. Glutamate and CGRP, which are implicated in migraine pathology, are co-expressed in trigeminal ganglion neurons, and are simultaneously released into the spinal cord following activation by peripheral stimuli [81; 102]. CGRP potentiates the excitatory effects of glutamate by binding to secondary neurons, causing the glutamate receptor N-methyl-D-aspartate (NMDA) to be brought to the cell surface. The upregulation of NMDA receptors induced by CGRP facilitates nociceptive signaling to the thalamus via the ascending pain pathway. GABA counters the effects of glutamate by reducing the excitability state of neurons and therefore blocking their activation via the descending pain modulation pathway [70]. If

neurons are not able to become activated, they will not transmit a signal to other neurons. The inhibitory effect of GABA is mediated via activation of GABA receptors expressed on primary and secondary neurons and glial cells in the trigeminal ganglion and spinal cord. For example, the reduced excitability state of primary and secondary trigeminal neurons caused by GABA is likely to involve the opening of a chloride ion channel in the cell membrane that makes the membrane in the cell more negative, which prevents generation of an action potential [55]. This cellular response opposes the effect of ligands that increase excitability, such as CGRP. In this way, GABA is not only responsible for promoting an anti-anxiety effect but can also function to inhibit pain signaling pathways [38].

GABA is synthesized in the cell body cytoplasm of a presynaptic neuron [100]. Glutamate decarboxylase (GAD) is the enzyme exclusively responsible for GABA synthesis. It functions to catalyze a decarboxylation reaction of glutamate to produce GABA and carbon dioxide. There are two isoforms of GAD: GAD 65 and GAD 67. GAD 65 is localized to the plasma membrane of GABAergic neurons, while GAD 67 is found primarily in the cytoplasm of neurons. Both isoforms are abundantly expressed in trigeminal ganglion neurons [99]. Synthesized GABA is stored in synaptic vesicles just below the plasma membrane of a neuron until vesicular release is initiated in response to a stimulatory signal [3; 38]. To facilitate exocytosis from the cell, the GABA-containing vesicle will align and fuse with the plasma membrane. This allows for GABA to be released outside of the cell into the synaptic cleft or extracellular matrix of the neuron. GABA can then bind to GABA receptors on another neuron or glial cell, or be taken up by transport proteins, and function to decrease neuronal excitability.

The GABA ligand can bind to 2 different GABA receptors: GABAA and GABAB [3; 38]. GABAA is an ionotropic or ligand-gated ion channel receptor and functions to allow an

influx of negatively charged chloride ions into the intracellular space when the GABA ligand is bound [63]. This increase in negatively charged ions lowers the excitability state of the cell by inhibiting generation of an action potential and the release of neurotransmitters and neuropeptides, as well as other pro-inflammatory molecules. GABAA consists of 5 subunits with two alpha groups, two beta groups, and one gamma group. Within the trigeminal ganglion, the GABAA receptor is expressed in both types of neurons [100].

GABAB, a G-protein coupled receptor comprised of the subunits GABAB1 and GABAB2 [53], is expressed on the cell surface of neurons, satellite glia [92], and Schwann cells [60]. Due to the nature of G-protein coupled receptors, activation of GABAB receptors may function as a preventative therapeutic while activation of GABAA receptors are likely to function as an abortive treatment [78]. Towards this end, the therapeutic benefit of non-invasive vagus nerve stimulation in aborting migraine was shown to involve activation of GABAA receptors within the spinal cord of rats [23]. Both subunits, GABAB1 and GABAB2, are structurally similar; they consist of an extracellular domain called the Venus flytrap domain (VFT), a 7 transmembrane domain called the heptahelical transmembrane domain (7TM), and a C-terminal tail [41]. The tails of the GABAB1 and GABAB2 subunits coil together within the intracellular space of the cell. The GABAB receptor only functions as a heterodimer; the two subunits cannot function without the other. Without GABAB2, GABAB1 is restricted to the endoplasmic reticulum and thus would not allow GABA to bind. GABAB2 allows for the translocation of GABAB1 to the cell surface and stabilization of the receptor. Because of this, GABAB2 can alter the binding affinity of GABA on the B1 receptor. The VFT domain in the B1 subunit is where the GABA ligand binds. This binding activates B2 to couple to an inhibitory Gi/o protein. This G protein will dissociate into $G\alpha$ and $G\beta\gamma$. The $G\alpha$ subunit inhibits

the activity of the signal transduction enzyme adenylate cyclase, which is positively regulated by CGRP. The inhibition of this enzyme activity leads to a reduction in cyclic AMP (cAMP) levels within the cell, inhibiting signal transmission, secretion, and decreasing excitability of the neuron. The $G\beta\gamma$ subunit activates inwardly rectifying K^+ (GIRK) channels, a subclass of the Kir family of channels, and inhibits voltage-gated calcium channels within neurons. Activation of GIRK channels results in positively charged potassium ions leaving the cell and decreases the neuronal membrane potential [11; 69; 93]. Inhibiting calcium channels blocks positively charged calcium ions from entering the cell, which is required for depolarization and generation of the action potential within a neuron.

Activation of the GABAB receptor within satellite glial cells causes stimulation of Kir channels [92]. Kir channels function to transport extracellular potassium across the plasma membrane into the cytoplasm to help maintain homeostasis. Dysfunction of this receptor is associated with neurons becoming hyperpolarized and hyperresponsive to external stimuli [98]. Together, these GABA receptor-mediated mechanisms lower the excitability state of a neuron and inhibit release of neurotransmitters and other signaling molecules in a neuroprotective manner. In addition to their inhibition of neuronal excitability, GABA receptors on satellite glia and Schwann cells function to decrease the excitability state of these cells, and thus disrupt the neuron-glia inflammatory loop and suppress the development and maintenance of peripheral sensitization.

Goal of the Study

In previous studies, we have shown that inclusion of GSE as a dietary supplement in the drinking water of rats inhibited trigeminal nociception in preclinical models of chronic TMD and

migraine [21; 24; 101]. The anti-nociceptive effect of GSE was blocked by injecting GABAB receptor and 5-HT_{3/7} receptor antagonists in the upper spinal cord/brainstem. However, administration of GABAA antagonists centrally did not block the inhibitory effects of GSE. In a more recent study, GSE was shown to also function via activation of the endocannabinoid system, which is important in facilitating descending pain modulation within the central nervous system. Although our research has shown that GSE can modulate pain signaling and the development of central sensitization in the spinal cord, it is not thoroughly understood how GSE modulates the activity of neurons and glial cells within the trigeminal ganglion that mediate peripheral sensitization.

In my thesis study, I wanted 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. Specifically, I wanted to investigate GSE's effect on CGRP secretion and peptide expression in trigeminal neurons. Additionally, I wanted to explore GSE's possible mechanism of action through the GABAergic system by determining GAD 65, GAD 67, and GABA receptor expression using immunocytochemistry. The main goal of my study was to test the hypothesis that GSE suppresses basal CGRP secretion via upregulation of the GABA receptors on trigeminal neurons and glial cells. I predicted that upregulation of GABA receptor expression in glial cells could play a critical role in inhibition of the continuation of peripheral sensitization mediated by increased CGRP-mediated neuron-glia communication. Results from my study will provide novel insights into the mechanisms of how GSE polyphenolic compounds modulation of GABA receptors could be useful as an adjunctive or alternative strategy for managing the pain and inflammation associated with chronic orofacial pain diseases.

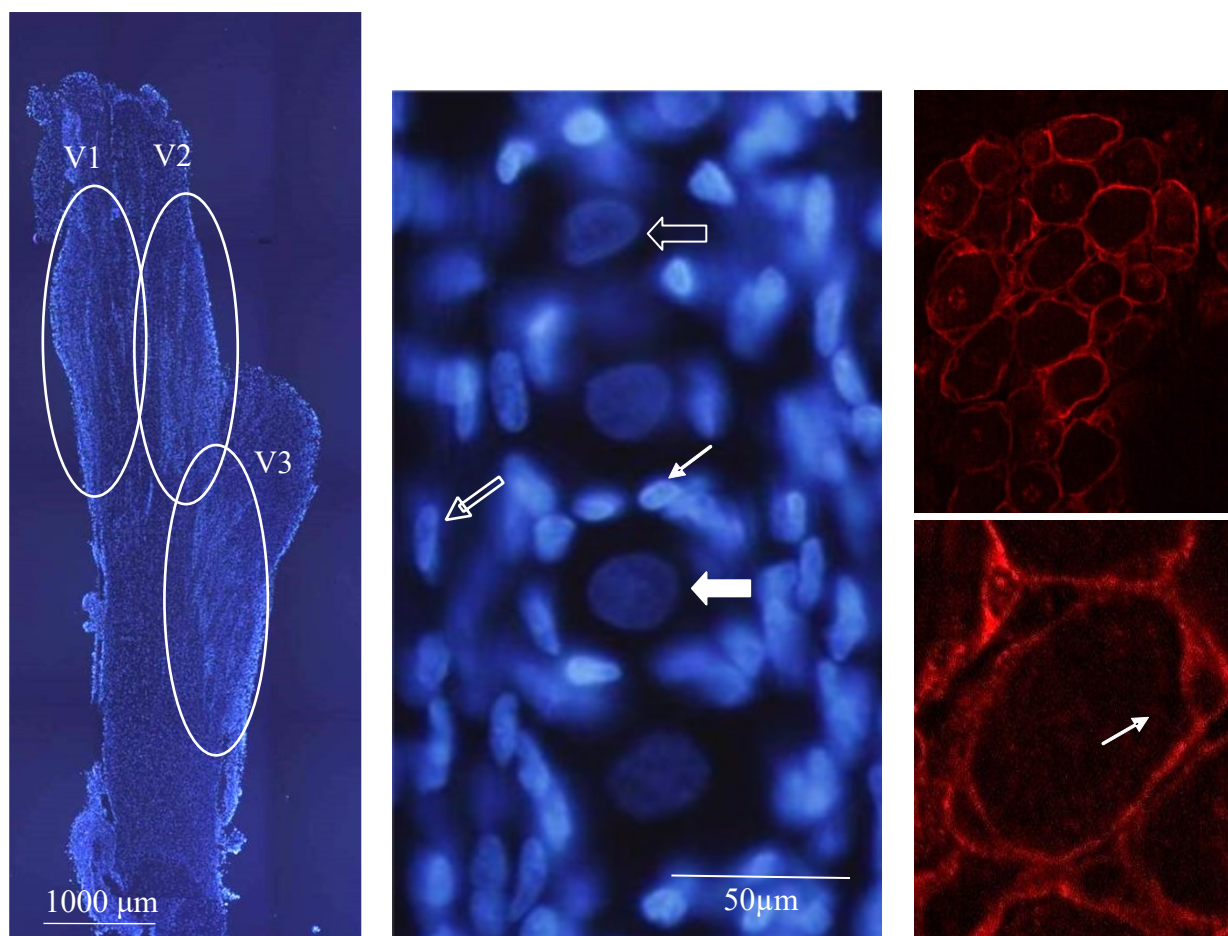


Fig. 1. Morphology of Trigeminal Ganglion. A representative image of 14 µm-thick section of the trigeminal ganglion stained with DAPI and the 3 branches labeled (left). A 400x image of functional units in a trigeminal ganglion with solid diagonal arrow pointing to the nucleus of a satellite glia cell, hollow diagonal arrow pointing to a Schwann cell, solid horizontal arrow pointing to an A δ fiber neuron, and a hollow horizontal arrow pointing to a C fiber neuron (middle). Kir 4.1 staining of satellite glial cells (right).

METHODS

Animals

Animal protocols were approved by the Institutional Animal Care and Use Committee at Missouri State University (IACUC protocol: 2020-06, see Appendix) and conducted in accordance with the guidelines of the National Institutes of Health. 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 neonatal pups were used to establish trigeminal ganglion cultures.

Establishment of Primary Trigeminal Ganglion Cultures

Primary cultures of trigeminal ganglia were established based on our previously published protocols [1; 10; 32; 34; 96]. 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. Once the tissue had settled, media was poured off and replaced with L-15 media containing 10 mg/mL Dispase II (Sigma-Aldrich) and 1 unit/ μ L RQ1 RNase-free DNase (Promega, Madison, WI). Ganglia and media were divided into two 15 mL tubes and rotated at 15 RPM in a 37°C incubator for 30 minutes. Tissues were observed for a noticeably feathery appearance after rotation, which was indicative of tissue digestion. Tubes were then centrifuged at 500 RPM for 2 minutes and the supernatant was poured off. Tissues were resuspended in 5 mL of L-15 plating

media. Tissues were dissociated by vigorous mechanical titration while carefully avoiding introduction of air into the media. Once the larger connective tissue had settled, the cloudy cell-containing media was removed and placed into a clean 15 mL tube. Media was centrifuged at 1300 RPM for 3 minutes 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 2.5 S nerve growth factor (Alomone Laboratories, Jerusalem, Israel). An antibiotic mixture of penicillin (100 units/mL) and streptomycin (100 µg/mL, Sigma-Aldrich) in addition to the antimycotic amphotericin B (2.5 mg/mL, Sigma-Aldrich) was added to the supplemented L15 media, which will be referred to as TG complete medium.

For immunocytochemistry studies, cells isolated from 3 trigeminal ganglia were plated on Poly-D-Lysine coated glass coverslips (Electron Microscopy Sciences, Hatfield, PA) in a 24-well plate (Corning Incorporated-Life Sciences, Durham, NC) and incubated at 37°C. For the secretion studies, cells isolated from 30 trigeminal ganglia were plated directly onto a 24-well plate in 250 µL of media and incubated at 37°C. For the toxicity studies, cells isolated from 12 trigeminal ganglia were plated directly onto a 96-well plate in 175 µL of media and incubated at 37°C. Cells for immunocytochemistry and toxicity were incubated with a 1:10,000 dilution of a 5 mg/mL stock solution (final concentration of 500 ng/mL) of MegaNatural®-BP grape seed extract (GSE, Healthy Origins, Pittsburgh, PA) prepared in cell water (BioWhittaker, Walkersville, MD) the day after dissection and plating.

Cultures were left to grow overnight after GSE was added. All cultures used for secretion, immunostaining, and toxicity studies were left to grow for a total of 2 days at 37°C

before processing the cell cultures for further analysis. All coverslips for immunostaining were fixed on day 3 using 4% paraformaldehyde (Sigma-Aldrich) and stored at 4°C. Following fixation, cells for immunocytochemistry were imaged at 200x on a Motic AE31 inverted light microscope with a Leica EC3 imager.

Testing Effect of GSE on Cell Viability

Following trigeminal ganglion culture setup and addition of a 1:1000 dilution of a 500 ng/mL GSE stock solution, viability of primary cultures after overnight incubation with GSE 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 hours at 37°C. After incubation, the absorbance was measured at 490 nm using a plate reader. Background absorbance was subtracted from signal and average values reported as absorbance at 490 nm.

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) before 250 µL of fresh HEPES-buffered saline (HBS: 22.5 mM HEPES, 135 mM NaCl, 3.5mM KCl, 1mM MgCl₂, 2.5 mM CaCl₂, 3.3 mM glucose, and 0.1% bovine serum albumin, pH 7.4) was added. Cells with fresh prewarmed HBS were left to incubate at 37° C for 1 hour before media was collected into a sterile 1.7 mL tube and labeled as “basal” secretion sample. Fresh prewarmed HBS (250 µL) was added to each of the wells. Some cells remained untreated (naïve), while other cells were

incubated for 60 minutes at 37°C with a final GSE concentration of 0.5 ng/μL. After 1 hour, media was collected into sterile 1.7 mL tubes labeled as “final” secretion samples. Cells remaining in the wells were rinsed with PBS twice before being scraped and collected into sterile 1.7 mL tubes labeled as “cell pellet” and later used for protein quantification.

The amount of secreted CGRP before and after GSE incubation was measured using a radioimmunoassay specific for CGRP (Phoenix Pharmaceuticals, Inc., Burlingame, CA). This assay was designed to detect levels of a specific peptide, rat CGRP, via competitive binding. An antibody specific to CGRP is added to the samples in a limited quantity. ¹²⁵I-CGRP, a radioactive synthetic version of CGRP, competes with the CGRP secreted from the trigeminal primary cultures into the media. A larger quantity of secreted CGRP competes for the radioactive CGRP's binding ability, thus producing a lower radiation measurement. A standard curve was generated that allows for quantification in the pg range of CGRP present in the samples. Radioactive counts were normalized to protein concentration, which was used to control for the number of plated cells per well. Total protein levels were determined using a standard Bradford protein quantification assay with absorbance measured on a Spectra Max plus 384 plate reader. Briefly, a standard protein curve ranging from 0 μg/μL to 3.5 μg/μL was made using bovine serum albumin (Sigma-Aldrich). Colorimetric Bradford reagent (Bio-Rad Laboratories, Inc., Hercules, CA) was added to all wells, and the absorbance value at 595 nm corresponds to protein abundance. The exact concentration in mg/mL is calculated based on the standard curve. Average means of CGRP secretion from naïve and GSE incubated samples from 6 independent experiments performed in duplicate were determined and reported as average fold change ± SEM relative to the average mean for naïve cultures, whose mean was set equal to one.

GSE Modulation of Protein Expression by Immunocytochemistry

Immunocytochemistry was performed to investigate changes in protein expression levels of naïve and GSE incubated primary trigeminal ganglion cultures. Primary cultures were incubated with a 0.5 ng/μl GSE solution. Primary cultures established as described in a prior section were treated with 5% donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA) + 0.1% Triton solution (Sigma-Aldrich) for 20 minutes to block non-specific antibody binding and permeabilize the cells. 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 Tween 80 solution (Fisher Scientific, Waltham, MA) two times followed by a rinse with PBS three times before being incubated with Alexa-Fluor conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). All secondary antibodies were prepared at a 1:200 dilution in 5% donkey serum and incubated for one hour at room temperature while being protected from light. At the end of secondary incubation, cells were rinsed with Tween twice and PBS three times. Coverslips 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 (Fisher Scientific) and secured with a clear coat of nail polish (L.A Colors, Ontario, CA).

All slides were imaged using a Zeiss AxioCam mRm camera (Carl Zeiss, Thornwood, NY) mounted on a Zeiss Imager Z1 fluorescent microscope within one week of staining and were stored at 4° C. A minimum of three 200x images were taken of each coverslip. 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. Intensity of the signal when set to the FITC green channel was measured by making a rectangle around each cell type of interest present in the image and subtracting the average background. Average means of the fluorescent intensities for each protein of interest in neurons and glial cells were determined and reported as average fold change \pm SEM relative to the naïve average, which was set to one. Each condition was repeated in triplicate in a minimum of 6 independent experiments.

Statistical Analysis

The following statistical analysis was performed for both immunocytochemistry and secretion data using SPSS Statistics Software version 24 (IBM, North Castle, NY). A Shapiro-Wilk test was used to evaluate normality of data distribution. For all data that was found to be normally distributed, a parametric independent t-test was performed. Levene's test was used to determine equal variances assumed. Independent t-tests were used to evaluate differences between naïve and GSE incubated conditions. For data that was not normally distributed, a nonparametric Mann Whitney U test was performed to determine differences between naïve and GSE incubated conditions. Data were considered significant if $p < 0.05$.

Table 1. Summary of Antibodies Used for Immunocytochemistry

Protein	Company; Catalog Number	Dilution	Incubation
CGRP	Abcam; Ab36001	1:2000	3 hr, room temp
β -tubulin	Abcam; Ab7751	1:5000	3 hr, room temp
GAD 65/67	Abcam; Ab11070	1:1000	3 hr, room temp
GABAA	Abcam; Ab72446	1:2000	3 hr, room temp
GABAB1	Abcam; Ab55051	1:2000	3 hr, room temp
GABAB2	Abcam Ab75838	1:2000	3 hr, room temp
Alexa-Fluor 488	JacksonImmuno Research; 711-545-152	1:200	1 hr, room temp
Alexa-Fluor 647	JacksonImmuno Research; 705-605-147	1:200	1 hr, room temp

RESULTS

Primary Culture Cell Types Mimic *In Vivo* Trigeminal Ganglion

Primary rat trigeminal ganglia cultures were used to study the effects of GSE on CGRP secretion and GABAergic-related protein staining. To confirm that cell culture populations resembled tissue cell composition, a fluorescent 4',6-diamidino-2-phenylindole (DAPI) stain was used to visualize the nucleus of all cells. The same 4 cell types (Schwann cells, satellite glial cells, A δ fiber neurons, and C fiber neurons) were found in whole trigeminal ganglia (Fig. 1) and my established primary cultures of trigeminal ganglia (Fig. 2). Neurons were identified using a DAPI stain and a light microscopy image based on their round nucleus with a size between 25 and 50 μm . Two types of neurons, A δ and C fiber were differentiated based on nuclear size. A δ are larger than C fibers. Any neuronal cell body larger than 35 μm was considered an A δ fiber neuron, while any neuronal cell body smaller than 35 μm was considered a C fiber neuron. Satellite glia were identified based on their small, more rounded nucleus. Schwann cells were identified based on their elongated nucleus and bipolar process morphology. In culture, neurons were present as approximately 14% (352 cells) of the total cell population. A δ neurons made up 2% (48 cells) and C fiber neurons made up 12% (291 cells) of total cells. Glial cells made up approximately 86% (2,126 cells) of the total cell population. Satellite glia cells and Schwann cells made up 59% (1,452 cells) and 27% (674 cells) of cell populations, respectively (n = 24).

To further verify that primary cultures mimic the *in situ* trigeminal ganglion tissue, the protein biomarkers calcitonin gene-related peptide (CGRP) and β -tubulin were detected using immunocytochemistry (Fig. 3, n = 6). β -tubulin, which is a subunit of the cytoskeletal microtubules and is localized in the cell bodies and processes, was used to show that cultured

neurons retained a neuronal phenotype. β -tubulin was expressed in all neurons and neuronal processes present in my cultures. CGRP, the proinflammatory neuropeptide of interest in this study, was expressed abundantly in a subset of neurons and neuronal processes in cultures. Costaining of β -tubulin and CGRP revealed that only a subset of A δ and C fiber neurons stained positively for CGRP. These data demonstrate neuronal expression of CGRP in less than half of the total neurons, which is similar to the level observed in trigeminal ganglion [57].

Characterization of Healthy Origins MegaNatural® BP-Grape Seed Extract

To ensure that overnight incubation with GSE was not causing toxic effects, cultured cells were incubated with GSE for 24 hours and cell viability was measured. Cell viability was unaffected with GSE incubation compared to an untreated naïve control but was greatly reduced by incubation with 10% DMSO (Table 2).

Effects of Grape Seed Extract on Basal CGRP Secretion

To determine if GSE could inhibit basal, or unstimulated, secretion of CGRP from trigeminal ganglion neurons, cultures were incubated in HBS containing 2.5 μ l of a 500 ng/mL GSE solution, resulting in a final concentration of 0.5 ng/ μ l (1.25 ng/well). As seen in Figure 4, GSE incubation for 60 minutes caused a significant decrease in basal CGRP secretion (124.6 ± 48.2 pg, $p = 0.025$, $n = 6$) when compared to the baseline level of CGRP (389.6 ± 83.8 pg). The level of CGRP in the HBS obtained from baseline and final naïve samples was measured as a control to ensure that the 60-minute incubation time and a full change of HBS did not significantly affect CGRP secretion. Naïve untreated cultures showed no difference between baseline and final CGRP secretion levels, with basal measurements being 248.3 ± 73.6 pg, and

final measurements being $221.6 \text{ pg} \pm 89.2 \text{ pg}$. Baseline CGRP secretion levels in wells prior to GSE incubation were measured in pg/mL of HBS normalized to protein concentration to ensure that the amount of baseline CGRP secretion was similar between the GSE incubated cultures and naïve cultures. The assumption is that the protein value directly correlates with the number of cells in each well. Although the average baseline levels of CGRP in GSE incubated wells were slightly higher than naïve baseline levels, the difference was not statistically significant between these conditions. Since the concentration of GSE used for the secretion experiments did not cause a change in cell viability 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.

CGRP

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 naïve levels (Fig. 5). 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 (101.1 ± 3.9 , $p = 0.459$, $n = 6$) when compared to naïve conditions (96.0 ± 5.2). Based on a summary of the average relative fold change \pm SEM in CGRP staining intensity of GSE incubated compared to naïve condition, GSE did not repress basal expression of CGRP.

GAD 65/67

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. 6). In cultured trigeminal ganglia, GAD 65/67 was detected abundantly in both A δ and C fiber neuronal cell bodies. The relative intensity of GAD 65/67 staining was significantly increased in neurons with overnight GSE incubation (48.1 ± 3.2 , $p = 0.033$, $n = 9$) compared to naïve conditions (38.8 ± 2.4). A summary of the average relative staining intensity of GAD 65/67 \pm SEM compared to naïve levels, whose mean was set equal to one, is shown below the images.

GABAA

Changes in expression of the GABAA receptor, a ligand-gated ion channel responsible for the influx of chloride ions when bound to GABA, in response to GSE incubation were investigated using immunocytochemistry (Fig. 7). In cultured trigeminal ganglia, GABAA was detected only in the cell body of A δ 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 (43.9 ± 4.8 , $p = 0.966$, $n = 6$) compared to naïve conditions (45.1 ± 3.4). A summary table of relative intensity of GABAA staining is shown below. A callout showing representative staining of a single neuron for naïve and GSE cultures is shown to better visualize GABAA expression. Based on immunostaining intensity measurements, cultures incubated with GSE did not effect GABAA receptor expression in trigeminal neurons.

GABAB1

Immunocytochemistry was used to investigate if GSE-mediated repression of basal CGRP secretion might correlate with 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 A δ and C fiber neurons (Fig. 8). Low level GABAB1 staining 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. A callout of a single GABAB1 stained neuron is shown to highlight the difference in staining intensity between the naïve and GSE conditions. A separate callout of a few glial cells is shown for naïve and GSE conditions to better visualize the increased GABAB1 expression in the cellular processes. Based on immunostaining intensity measurements, GSE incubated cells showed a significant increase of GABAB1 staining intensity in the neurons (65.2 ± 5.9 , $p = 0.013$, $n = 7$) compared to naïve control levels (43.1 ± 3.4). 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 (20.5 ± 1.0 , $p = 0.006$, $n = 6$) when compared to naïve control levels (14.5 ± 1.4).

GABAB2

Changes in expression of the GABAB2 receptor, which is responsible for the 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. 9). Staining for GABAB2 showed no intensity

change with GSE incubation (52.0 ± 2.6 , $p = 0.628$, $n = 6$) in the neurons compared to naïve levels (50.0 ± 3.0). However, the intensity of GABAB2 immunostaining was significantly increased in satellite glia and Schwann cells in response to GSE (11.7 ± 1.0 , $p = 0.016$, $n = 6$) when compared to levels in the naïve condition (7.3 ± 0.5). A callout of a single GABAB2 stained neuron is shown for naïve and GSE conditions to emphasize that there was no change in staining intensity in either neuronal cell type. A separate callout of a few glial cells is shown for both conditions to clearly demonstrate the upregulation of GABAB2 expression in the cytoplasm and processes of each glial cell type. Based on average relative immunostaining intensity measurements, GSE incubated cells 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.

Table 2. Summary of Cell Viability After GSE Incubation

Sample	Absorbance	Fold Change
Naïve	0.425	1.000
GSE 24 hr	0.488	1.146
10% DMSO 24 hr	0.290	0.648

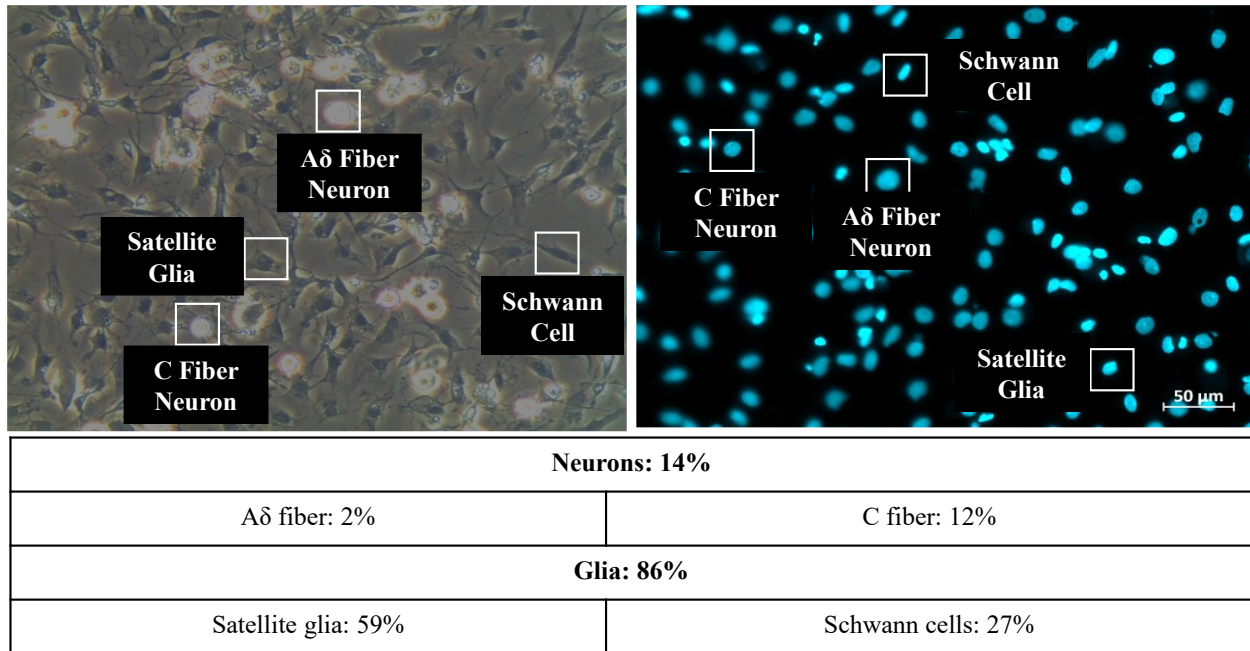


Fig. 2. Characterization of Cell Types in Primary Cultures. A representative light microscopy image at 200x with labeled cell types (left). A representative image of DAPI stained primary cultures imaged at 200x with labeled cell types (right). The relative abundance of each cell type as a percentage of the total cell population is summarized in the table.

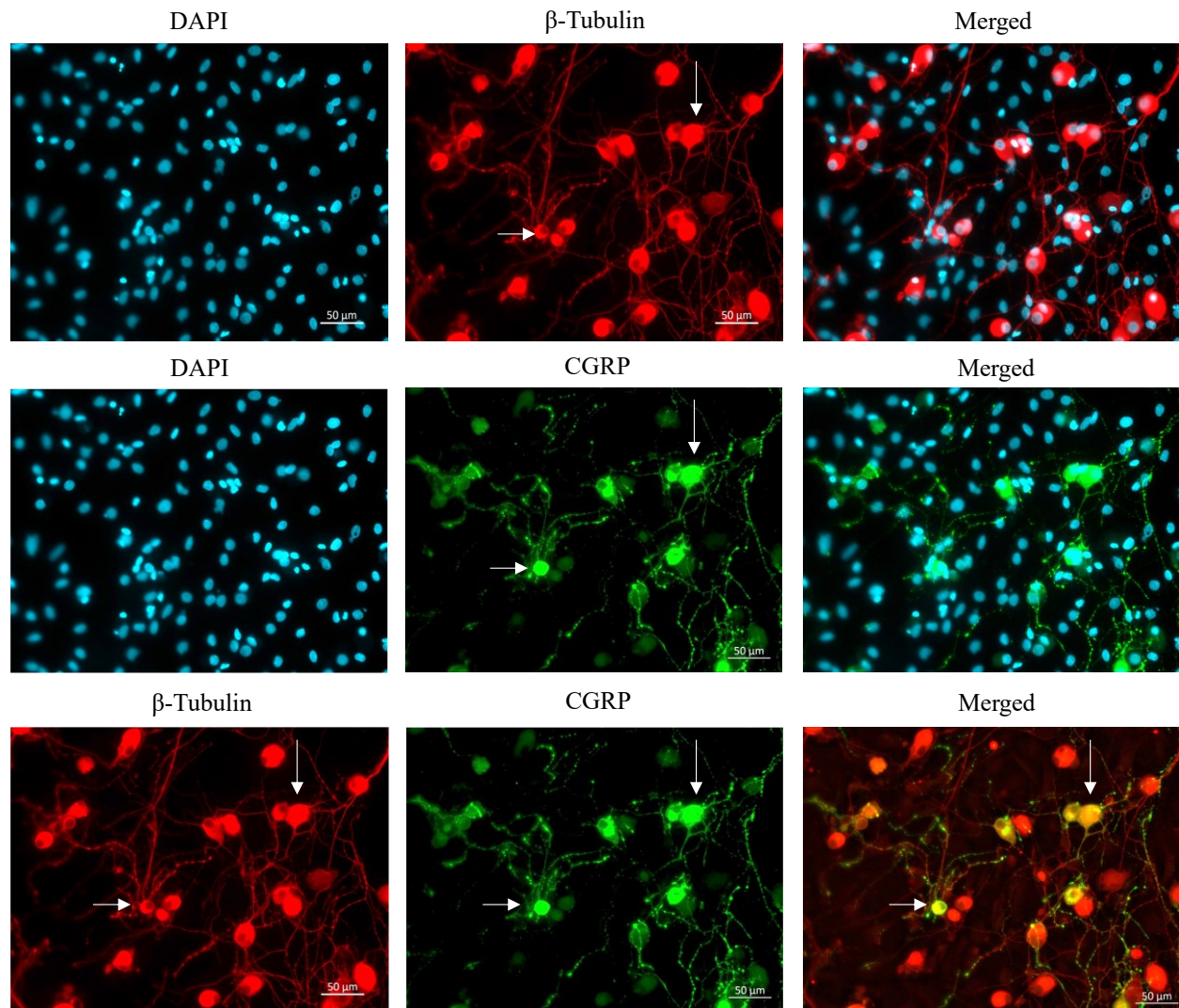


Fig. 3. Expression of Neuronal Biomarkers in Primary Cultures. All representative images are at 200x magnification, with a horizontal arrow indicating a C fiber neuron and a vertical arrow indicating an A δ fiber neuron. DAPI and β -tubulin costaining in C fiber and A δ fiber neuronal cell bodies and processes (top row). DAPI and CGRP costaining in C fiber and A δ fiber neuronal cell bodies (middle row). β -tubulin and CGRP costaining showing that only a subpopulation of neurons abundantly express CGRP.

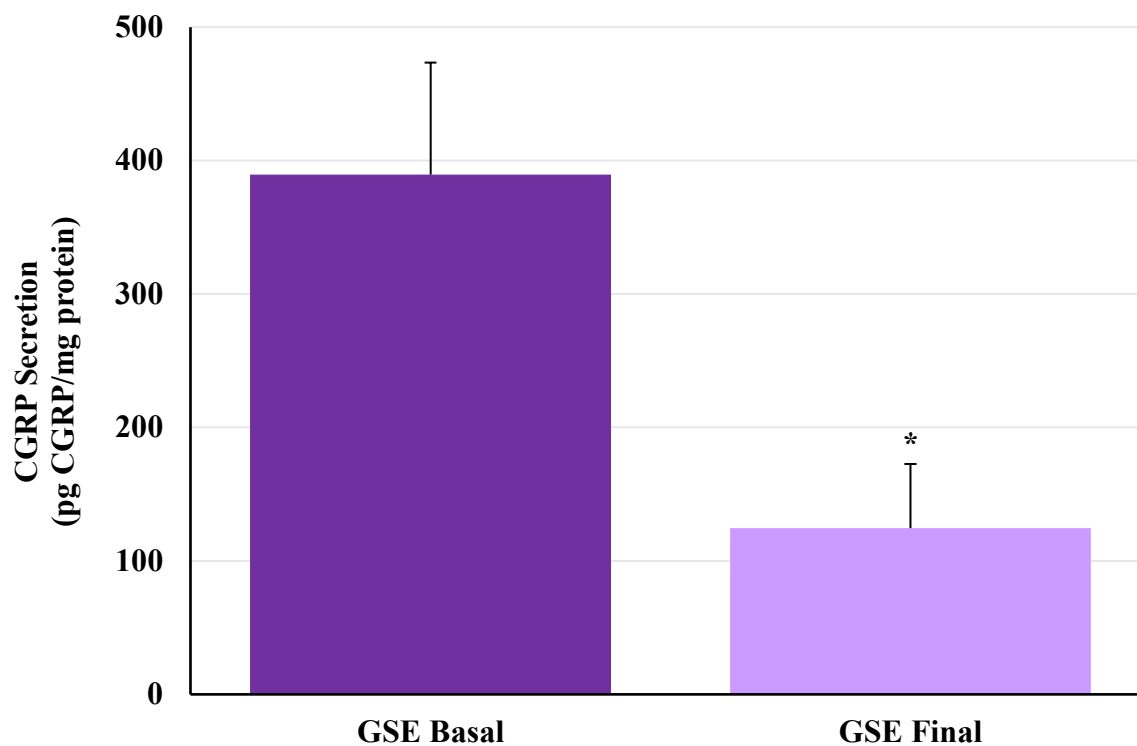


Fig. 4. GSE Repression of Basal CGRP Secretion from Trigeminal Ganglion Neurons. The amount of CGRP secreted into the culture media under unstimulated basal condition and following 60-minute incubation with GSE was determined by radioimmunoassay. Data is reported as pg CGRP normalized to total protein level in the well. * = $p < 0.05$, $n = 6$ done in duplicate for each condition.

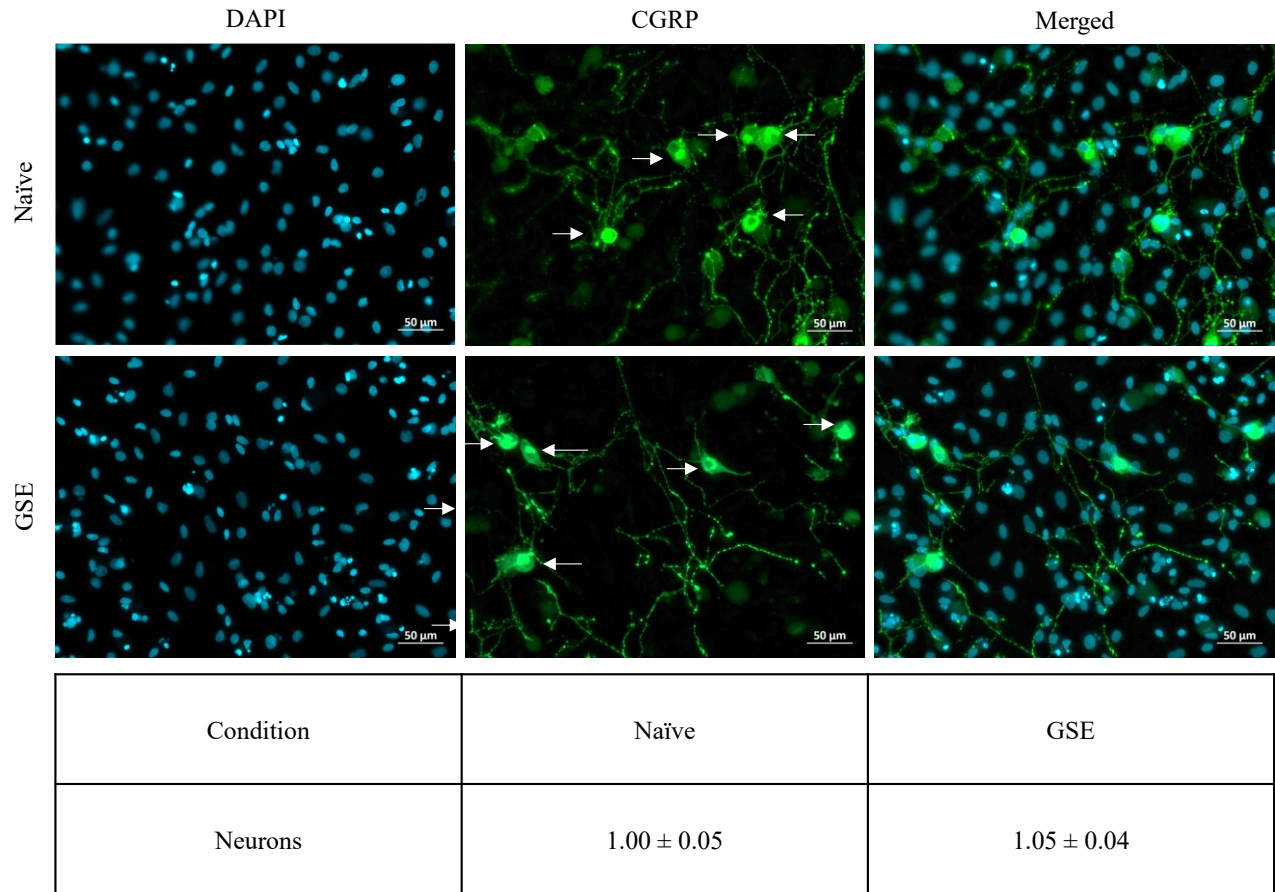


Fig. 5. CGRP Immunostaining Levels in Primary Cultures Does Not Change with GSE Incubation. All representative images are at 200x magnification. DAPI, CGRP, and merged staining of naïve (top) and GSE (bottom) conditions. White arrows indicate neuronal cell bodies abundantly expressing CGRP. A summary table of average relative intensity \pm standard error of the mean is shown below. $n = 6$ done in triplicate for each condition.

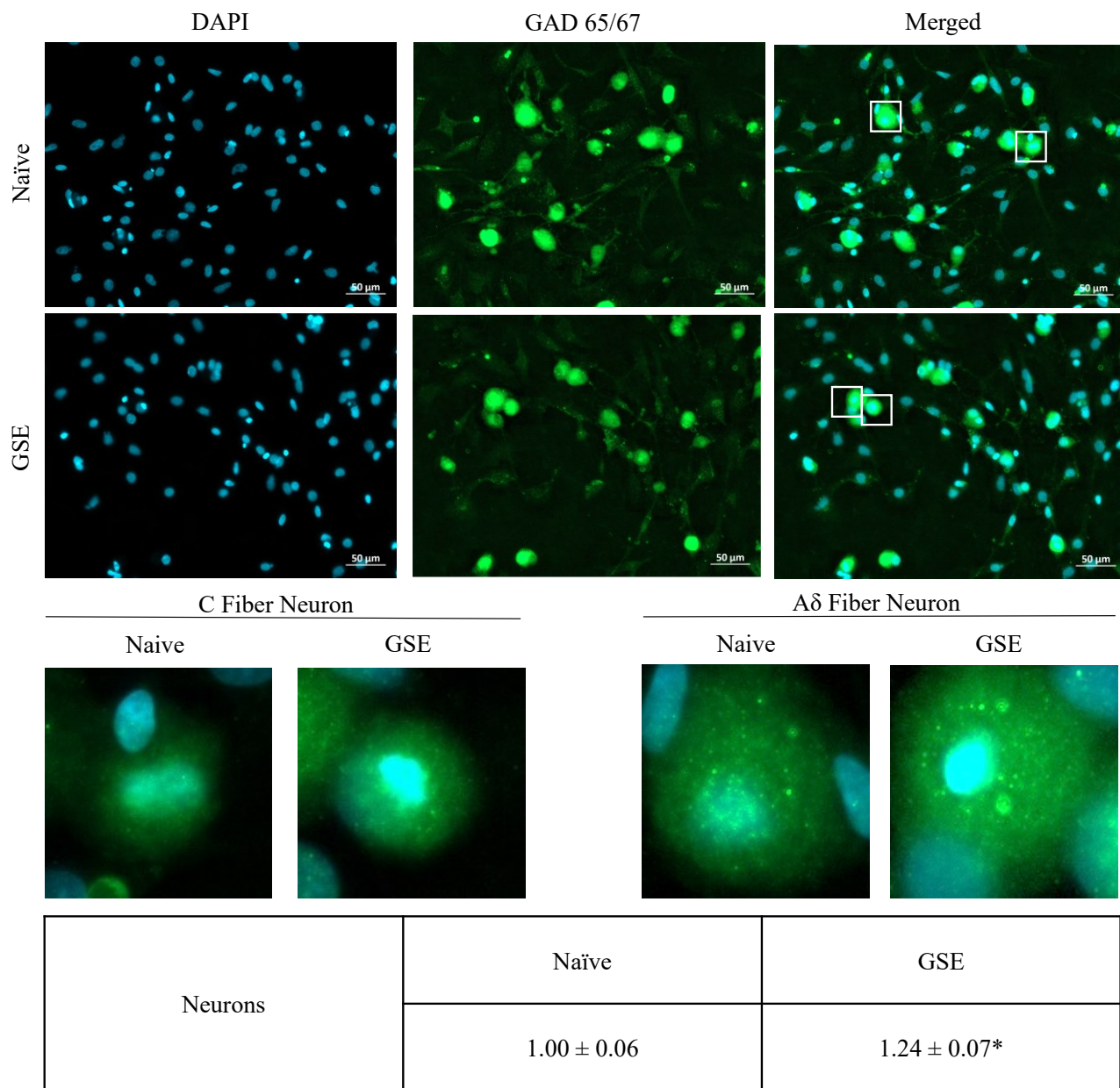


Fig. 6. GAD 65/67 Immunostaining Levels in Primary Cultures Increases in Neurons with GSE Incubation. All representative images are at 200x magnification. DAPI, GAD 65/67, and merged staining of naïve (top) and GSE (bottom) conditions. Callouts are shown for both neuronal cell types, C fiber and A δ fiber. A summary table of average relative intensity \pm standard error of the mean is shown below. * = $p < 0.05$; $n = 9$ done in triplicate for each condition.

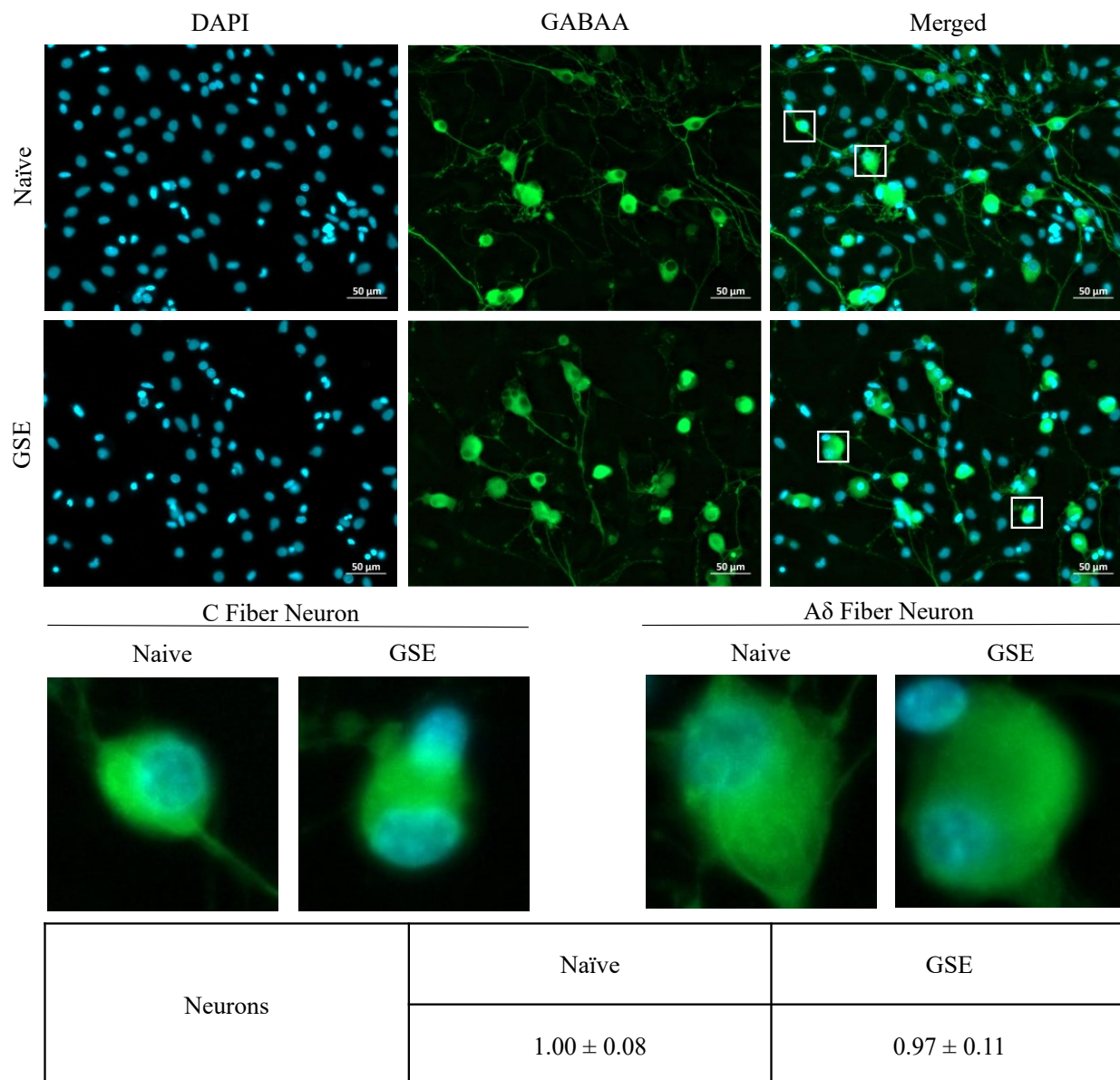


Fig. 7. GABAA Immunostaining Levels in Primary Cultures Shows No Change in Neurons with GSE Incubation. All representative images are at 200x magnification. DAPI, GABAA, and merged staining of naïve (top) and GSE (bottom) conditions. Callouts are shown below for both neuronal cell types, C fiber and Aδ fiber. A summary table of average relative intensity \pm standard error of the mean is shown below. $n = 6$ done in triplicate for each condition.

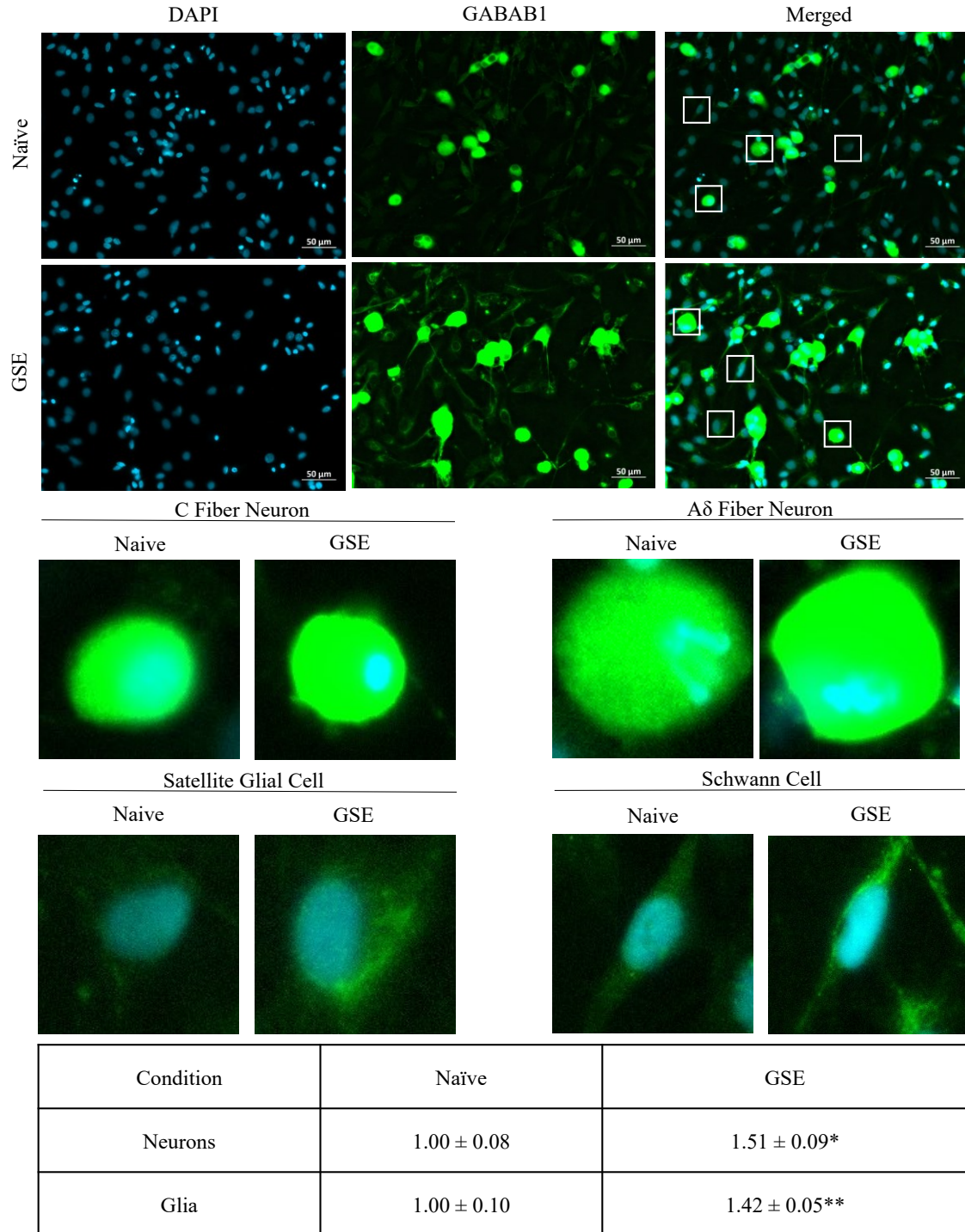


Fig. 8. GABAB1 Immunostaining Levels in Primary Cultures Increases in Neurons and Glia with GSE Incubation. All representative images are at 200x magnification. DAPI, GABAB1, and merged staining of naïve (top) and GSE (bottom) conditions. Neuron and glia callouts are shown for each of the four cell types. A summary table of average relative intensity \pm standard error of the mean is shown below. * = $p < 0.05$; ** = $p < 0.01$; $n = 7$ for neurons, $n = 6$ for glia done in triplicate for each condition.

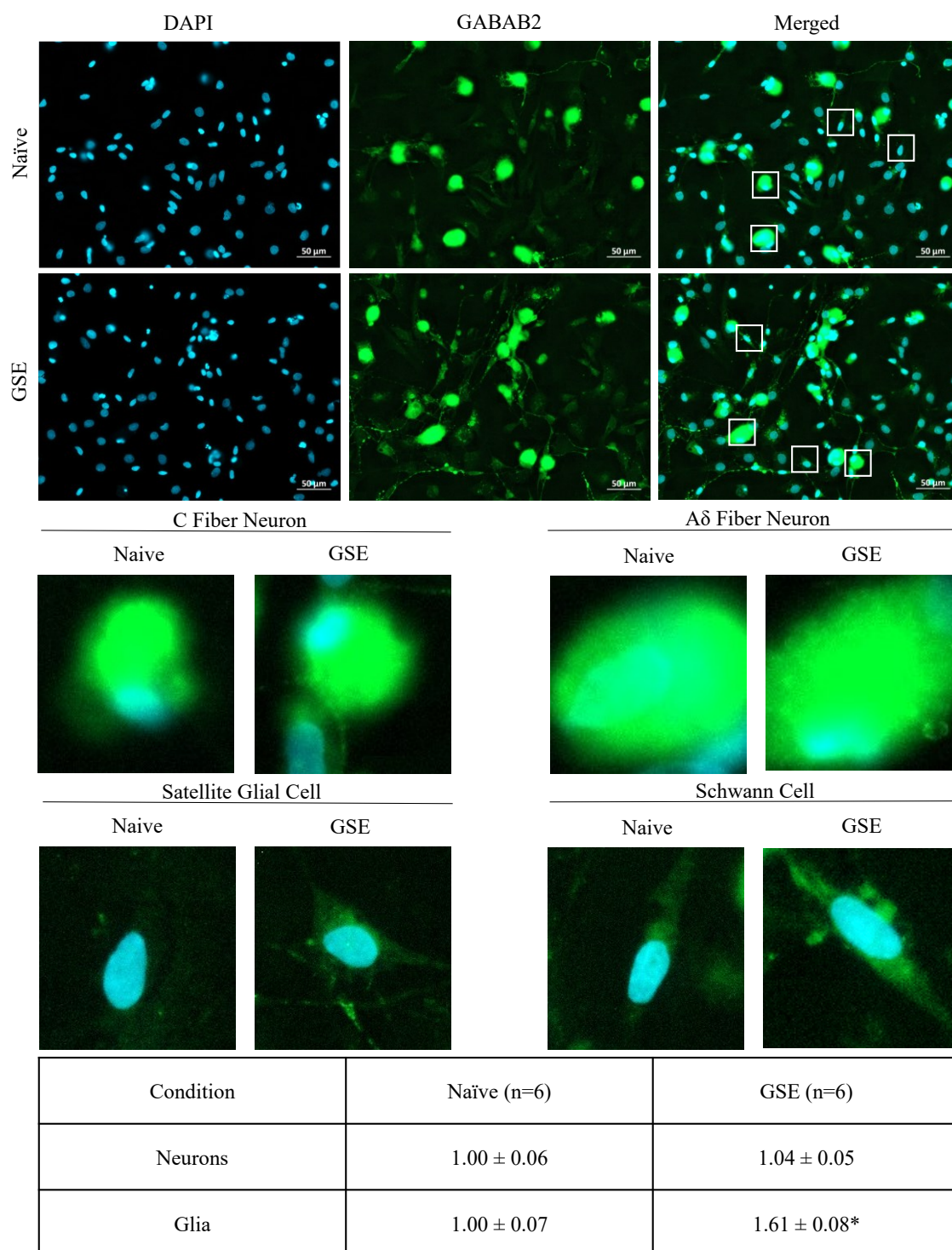


Fig. 9. GABAB2 Immunostaining Levels in Primary Cultures Increases in Glia, No Change in Neurons with GSE Incubation. All representative images are at 200x magnification. DAPI, GABAB2, and merged staining shown for naïve (top) and GSE (bottom) conditions. Neuron and glia callouts are shown for each of the four cell types. A summary table of average relative intensity \pm standard error of the mean is shown below. * = $p < 0.05$; n = 6 done in triplicate for each condition.

DISCUSSION

Results from my study show 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/ μ L for 60 minutes significantly inhibited basal CGRP secretion. However, CGRP expression within the neuronal cell body of A δ 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 leakage of cytoplasmic contents because of cell death. These data demonstrate that while this concentration of GSE is sufficient to repress basal CGRP release from neurons, it is not sufficient to cause a decrease in overall CGRP levels following an overnight incubation. This finding may not be surprising given that CGRP is the most abundant neuropeptide expressed in trigeminal ganglion neurons and thus longer incubation times and higher GSE concentrations may be required to see a corresponding change in protein levels via repression of mRNA synthesis [52]. Alternatively, GSE may only function at the level of secretion to decrease the effects of CGRP. The ability of GSE to inhibit constitutive secretion of CGRP from cultured trigeminal ganglion neurons is novel. Results from prior studies with the anti-migraine agents sumatriptan [34], topiramate [33], botulinum toxin type A [29], and a cocoa extract [1] did not suppress basal CGRP secretion from trigeminal neurons, but only inhibited stimulated CGRP release. Hence, GSE appears to function in a unique way to modulate the excitability state of trigeminal neurons by suppressing constitutive CGRP secretion.

The demonstration that GSE inhibits basal CGRP release from trigeminal ganglion neurons has 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 [28; 62]. Furthermore, my findings support the notion that GSE may be beneficial as a nutraceutical since CGRP release from trigeminal neurons is implicated in prevalent orofacial pain diseases, including migraine and TMD. Suppression of basal CGRP secretion could offer a promising alternative or adjunctive therapeutic by countering the pro-inflammatory and nociceptive effects of CGRP. My *in vitro* findings agree with prior results from our lab that had shown GSE works *in vivo* to successfully inhibit the development of central sensitization and chronic orofacial pain signaling in preclinical models of TMD and migraine [18; 21; 24; 101]. In those studies, dietary inclusion of GSE (0.5% w/v in the drinking water), which was sufficient to inhibit trigeminal nociception, was found to mediate its inhibitory effects via activation of the descending inhibitory pain modulation pathway. GSE functioned by activating serotonergic, GABAergic, and endocannabinoid receptors expressed on neurons and glial cells in the upper spinal cord. Taken together, I propose that GSE inhibits development of peripheral and central sensitization of the trigeminal system via suppression of CGRP signaling, which is implicated in migraine and TMD pathology. Based on my initial results and our lab's prior findings on GSE, I wanted to test the hypothesis that GSE repression of CGRP secretion from primary neurons is mediated via increased synthesis of GABA and activation of GABA receptors.

In support of my hypothesis, I found 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 [56]. Under basal, unstimulated conditions, GAD 65/67 immunostaining was readily detected in both A δ 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 [12]. 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. My 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 [49] and that GAD 65 and GAD 67 are colocalized with CGRP [68]. To my knowledge, this is the first evidence of a polyphenolic-enriched extract promoting increased expression of GABA producing enzymes in neuronal cells.

GSE also stimulated expression of the GABAB1 receptor subunit, which is responsible for binding GABA and then forming a functional complex with the GABAB2 subunit [38]. The GABAB1 receptor subunit was significantly upregulated in trigeminal ganglion A δ 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 release of CGRP from trigeminal neurons can be caused via activation of the stimulatory G protein-PKA-cAMP coupled pathway, and this pathway is implicated in the development of peripheral sensitization [17; 22]. Thus, the GSE-mediated increase in the functional expression of the GABAB receptor via upregulation of GABAB1 in trigeminal neurons could directly counter the cellular effects of other stimulatory agents such as nitric oxide and cytokines that facilitate CGRP release [10; 33]. 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 [18; 21; 24; 101].

Increased activation of the GABAB receptors would promote potassium efflux, inhibition of calcium influx, and a decrease in cyclic AMP levels in the neurons, resulting in a lower neuronal excitability state. Hence, a greater inflammatory stimulus would be required to cause depolarization and activation of the A δ and C fiber neurons. Additionally, GABAB receptor activation is reported to promote glial cells to uptake extracellular potassium, which would further promote a decrease neuronal excitability (see section below). Through these inhibitory G protein-coupled mechanisms, activation of the GABAB receptors would lead to a lower excitability state of the neuron and a quieting of the trigeminal system. Furthermore, these GSE-mediated cellular events on the GABAergic system would be expected to inhibit the release of neurotransmitters and neuropeptides such as CGRP from primary trigeminal neurons as discovered in my study.

I next wanted to investigate the neuronal expression of other GABAergic proteins such as the GABAA receptor in response to GSE. 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 [3; 63]. Somewhat surprisingly, GSE incubation overnight 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. Interestingly, activation of the GABAA receptor, but not GABAB, was found to play an important role in the anti-nociceptive effect of non-invasive vagus nerve stimulation (nVNS) in a preclinical chronic migraine model [23]. 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, it is now appreciated that glial cells play an important role in modulating the excitability state of neurons and the development of peripheral sensitization [62]. For this reason, I next looked at the expression of GABAB receptors in glial cells, since GABAB is the only GABA receptor reported to be expressed in glia [91; 92; 99].

In my study, I found that 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. Given the important role of glial cells in modulating the excitability state of primary sensory neurons, 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 [28]. 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 [58; 97]. Thus, inhibiting CGRP-mediated cellular events in glial cells would suppress the development and maintenance of the neuron-glial 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 [31; 43].

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 [26]. 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 A δ and C fiber neurons to cause cellular changes associated with allodynic pain signaling. Based on my findings, I propose that upregulation of the GABAB receptor subunits in Schwann cells would inhibit the synthesis and release of nitric oxide, which is stimulated by CGRP. To our knowledge, this is the first evidence of a nutraceutical modulating GABAB subunit receptor expression in peripheral glial cells.

Previous *in vivo* inhibitor studies from our lab support the idea that treatment with the nutraceutical GSE acts to decrease orofacial pain via activation of the GABAergic system within the upper spinal cord [24]. Thus, GSE would mediate suppression of central sensitization and inhibit nociceptive signaling via the secondary neurons to the thalamus. However, it is known that GABA receptors are also expressed on the processes of the trigeminal primary neurons that project into the dorsal medullary horn of the spinal trigeminal nucleus in the upper spinal cord. My data supports the notion that GSE's inhibitory effect on pain signaling likely also involves upregulation of the GABA producing enzymes GAD 65 and GAD 67 and activation of GABAB1 expression on primary trigeminal neurons (Fig. 10). Upregulation of the GAD enzymes in both A δ and C fiber neurons would provide an increased endogenous source of GABA both in the trigeminal ganglion and spinal cord. This increased production of GABA would directly lead to decreased CGRP secretion in nearby neurons by activating their GABAA and GABAB

receptors, thus establishing a negative feedback loop involving modulation of both neurons and glia to restore and maintain homeostasis. Further, GSE-mediated upregulation of the GABAB1 receptor subunit in neurons would inhibit CGRP secretion. In addition, upregulation of GABAB1 and GABAB2 subunits in both satellite glia and Schwann cells would suppress the stimulatory effects of CGRP that promote and sustain the inflammatory loop implicated in migraine and TMD pathology. In summary, I propose that the inhibitory effects of GSE on pain signaling in preclinical models of migraine and TMD involves modulation of GABAergic system to suppress the development of peripheral and central sensitization of trigeminal neurons. To my knowledge, these are novel findings that support the neuroprotective role of GSE and the potential health benefit of using GSE as a complementary or adjunctive approach for the clinical management of migraine and TMD and possibly other types of orofacial pain.

Findings from our lab have previously shown 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 [24; 101]. The cellular effects of GSE include 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 [18]. My results extend our knowledge of how GSE functions as a neuroprotectant 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. If GSE functions similarly in humans, then inclusion of this nutraceutical as a daily supplement would likely enhance the therapeutic benefit of currently used drugs. GSE may enhance the effect of the GABAB agonist Baclofen, which is the only

clinically available drug that targets the GABAB receptor [38]. Combining a nutraceutical with a pharmaceutical, such as baclofen, could offer a more effective treatment strategy. Findings from my study provide evidence that GSE can act peripherally to exert effects centrally. A current preventative treatment for chronic migraine is to use a monoclonal antibody directed against CGRP or its receptor to inhibit its downstream pro-inflammatory effects on neuron and glial cells [95]. Since CGRP monoclonal antibodies cannot effectively cross the blood brain barrier, this is another therapeutic in which its disease modifying effects may be improved by inclusion of GSE as a dietary supplement. Based on findings from my study, inclusion of GSE would function in neurons along with associated satellite glia, and nearby Schwann cells within the trigeminal system to suppress the nociceptive effects of CGRP. Given that elevated levels of CGRP are implicated in TMD pathology, it is highly likely that GSE would be clinically beneficial in this orofacial pain condition that is frequently comorbid with migraine [19].

The results of my study raise many interesting questions with respect to how GSE is functioning. Clearly, further investigations are needed to more fully understand the diverse cellular and molecular changes mediated by GSE. Continuing my *in vitro* studies, I would like to utilize immunocytochemistry using antibodies specific for the GAD 65 and GAD 67 enzymes to determine if both proteins are being upregulated in response to GSE. Alternatively, I could perform western blot analysis to determine changes in protein expression of the membrane bound GAD 65 and cytosolic GAD 67 enzymes. Also using immunocytochemistry, I would like to look at expression levels of GLAST and GLT-1 to see if GSE upregulates these proteins peripherally as well as centrally. It would be interesting to identify which class of polyphenols was most responsible for the inhibitory activity of GSE. I could fractionate GSE using different solvents to isolate specific components and then evaluate those compounds in primary trigeminal

cultures. If given the opportunity, I would like to use fluorescent microscopy to investigate GSE mediated changes in intracellular ion levels under basal, stimulated, and GSE conditions. For example, cells could be loaded with specific molecules that allow for quantification of ion levels of calcium, potassium, sodium, and chloride, which would provide novel information about how GSE specifically modulates the excitability state of neurons and glial cells. Another way to investigate further the mechanism of action of GSE *in vitro* would be to perform transient transfection of the primary cultures with different reporter plasmids to determine if GSE inhibited promoter activity of genes known to be stimulated by CGRP, such as PKA and MAP kinases. Results from these studies would provide evidence if GSE is suppressing the downstream effects of CGRP at the transcriptional level. Additionally, a logical next step would be to utilize our *in vivo* preclinical models of migraine and TMD and immunohistochemistry to study the modulatory effects of GSE on the expression of GAD 65, GAD 67, and GABAB receptor subunits in the trigeminal ganglion and upper spinal cord.

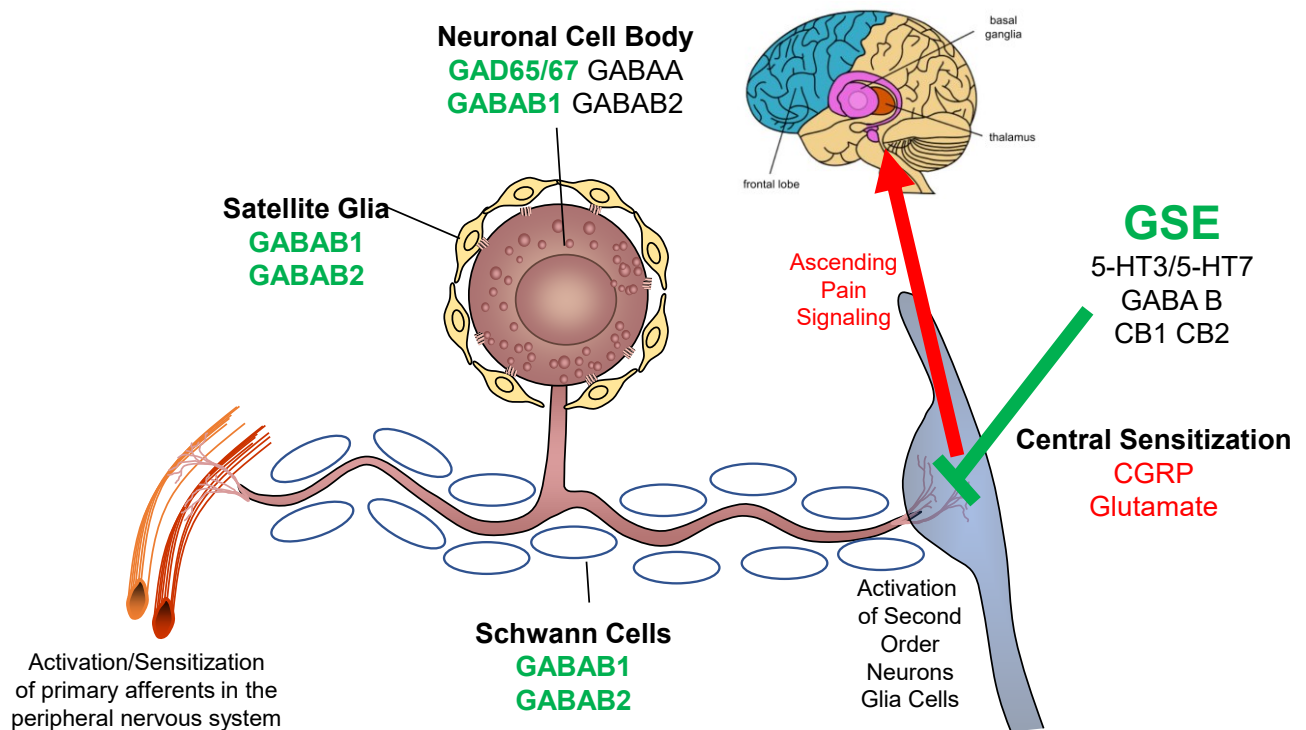


Fig. 10. Graphical Abstract. A graphical depiction of proteins expressed in the trigeminal system, with proteins being upregulated by GSE shown in green. See text for more details.

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Appendix: IACUC Approval Letter

Shown below is the letter of approval from the IACUC committee for conducting this research.



June 1, 2022

RE: IACUC protocol 2020-06

Sophia Antonopoulos,

IACUC protocol #2020-06 entitled "Use of Central Agonists and Antagonists to Investigate Mechanisms and Treatments of Orofacial Pain" was approved by the committee on April 22, 2020 and expires 4/21/2023.

The protocol reflects that you were approved to work with Dr. Paul Durham on this project at that time.

Thank you and if you need anything in the future regarding this protocol, please contact me either via email (johnnapedersen@missouristate.edu) or at 417-836-3737.

Sincerely,



Johnna Pedersen
IACUC Administrator

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