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Dietary Inclusion of Enriched Chicken Bone Broth Prevents Trigeminal Sensitization Meditated by Early Life Stress

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**DIETARY INCLUSION OF ENRICHED CHICKEN BONE BROTH PREVENTS
TRIGEMINAL SENSITIZATION MEDITATED BY EARLY LIFE STRESS**

A Masters Thesis

Presented to

The Graduate College of

Missouri State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science, Biology

By

Orion Peterson

August 2018

DIETARY INCLUSION OF ENRICHED CHICKEN BONE BROTH PREVENTS TRIGEMINAL SENSITIZATION MEDITATED BY EARLY LIFE STRESS

Biology

Missouri State University, August 2018

Master of Science

Orion Peterson

ABSTRACT

Early life stress is considered a risk factor for development of migraine, which is a prevalent painful neurological disorder involving sensitization and activation of trigeminal neurons, and irritable bowel syndrome, a condition often comorbid with chronic migraine. The focus of my study was to determine the effects of early life stress and dietary inclusion of enriched chicken bone broth on trigeminal nociceptor sensitization and the gut microbiota. Adult Sprague-Dawley male “sender” rats subjected to primary traumatic stress were placed next to breeding or pregnant female rats that served as “receiver” rats (secondary traumatic stress) and in proximity to the offspring after weaning. Unstressed and stressed young adult offspring were tested for changes in basal nocifensive response to mechanical stimulation and following exposure to a reported migraine trigger, the pungent odor from an extract of the California bay leaf. Early life stress increased basal trigeminal nocifensive responses in females and promoted a persistent sensitized state of trigeminal nociceptors that were activated by the pungent odor in both sexes. Female animals exhibited a greater level of basal sensitivity and enhanced activation when compared to males. Gender differences were also observed in fecal and cecum microbiota at the genus level between naïve and stressed rats. Dietary inclusion of bone broth at the time of weaning was sufficient to inhibit basal and triggered nociception. In summary, my findings support the notion that early life stress promotes a sensitized trigeminal system associated with increased risk of migraine, and dietary supplementation with chicken bone broth may provide an effective non-pharmacological method for reducing migraine risk.

KEYWORDS: stress, microbiota, nociception, trigeminal, bone broth, migraine

This abstract is approved as to form and content

Paul L. Durham, PhD
Chairperson, Advisory Committee
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Approved:

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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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INTRODUCTION

Stress

Types of Stress and Health Implications. Stress is defined as any unpleasant mental or physical strain on an individual. When an individual experiences stress, several physiological changes occur. The sympathetic nervous system, which is responsible for the fight or flight response, triggers the release of stress hormones, which cause elevations in heart rate and blood pressure, stimulates sweating, inhibits gastric emptying, and increases colonic motility (Tsigos et al., 2000). Once the stressor is eliminated or minimized, the parasympathetic nervous system functions in a compensatory manner to relax the body, slow heart rate, and restore stomach and intestinal activity (Tsigos et al., 2000). There are multiple recognized types of stress depending on the type of stressor and the individual being exposed to the stress. Acute stress is when an individual is exposed to a stressor infrequently, homeostasis is restored following the stress, and there are no long-term negative effects on an individual. Chronic stress occurs when an individual is exposed to stress for a longer duration or the stress is recurrent, unrelenting, and causes maladaptive physiological changes within an organism. Chronic stress is often experienced in individuals with high demanding jobs, poor sleeping habits, and clinically diagnosed anxiety disorders (Guan et al., 2017; Hall et al., 2015). While acute stress is associated with increased resiliency, prolonged, unmanaged stress can promote development of medical conditions and even chronic disease states. Chronically stressed individuals that are left untreated will have elevated levels of stress hormones systemically that promote inflammation, may exhibit detrimental behaviors like grinding

teeth that can lead to development of temporomandibular joint disorder (TMD), and are likely to experience persistent tension in their neck and shoulder muscles, which is implicated in migraine and headache pathology (Fillingim et al., 2013; Houle and Nash, 2008). Migraine is a prevalent neurovascular disorder that affects over 20 percent of women and over 10 percent of men at least once in their lives (Weatherall, 2015), while tension headache is commonly experienced by children and adults during stressful times (Chowdhury, 2012; Loder and Rizzoli, 2008). These debilitating pathophysiological disorders can function as risk factors for the development of other complex conditions such as cardiovascular disease, depression, irritable bowel syndrome, and diabetes (Mariotti, 2015; Qin et al., 2014).

Most of the human population is subjected daily to physical, emotional, social, and psychological stressors that were not present in the world of our ancestors when our nervous and digestive systems were evolving. While humans are most well adapted to dealing with physical stress, the impact of other types of stress are often more difficult to manage and, if chronic, can promote a disease state. The sympathetic nervous systems' fight or flight response serves as a mechanism to protect humans against the threat of physical harm. Due to its short-term nature, physical stress is rarely classified as chronic stress (Winston and Sarna, 2016). However, psychological stress, like job performance stress or social anxiety, is more likely to cause maladaptive changes in humans due to the inability to effectively eliminate the stressor and prevent persistent activation of the sympathetic nervous system (Won and Kim, 2016). Unfortunately, unmanaged chronic stress due to consistent psychological and emotional strain from mental and social

pressures is associated with a greater risk of developing diseases associated with the nervous and digestive systems (Winston and Sarna, 2016).

The type of stress that is most obvious and well-studied would be considered primary traumatic stress. Primary traumatic stress is experienced when an individual is put directly into a situation that elicits an immediate physiological stress response. In humans, examples of primary traumatic stress would be a physical or verbal confrontation, struggling to tread water if one cannot swim, and public speaking.

Secondary traumatic stress, which is also referred to as compassion fatigue, is reported by the National Child Traumatic Stress Network as emotional duress that results when an individual hears about or witnesses the firsthand traumatic experiences of another individual. While this individual did not directly experience the stressor, they are experiencing a similar sympathetic stress response by hearing or witnessing what happened to someone else. Long term exposure to this type of stress, seen in people that work in the social services sector, may lead to stress-related mental and physical conditions such as anxiety and depression, obesity, cardiovascular disease, irritable bowel syndrome, as well as more chronic pain conditions including migraine and TMD (Smith Hatcher et al., 2011). While most animal studies conducted on stress and chronic pain focus on male models, females are at a higher risk for developing neck muscle tension and jaw injury, which can lead to migraine, TMD, and chronic pain conditions (Bagis et al., 2012). Thus, known sexual physiological differences in the structure and function of particular brain regions mediated by increased estrogen levels after puberty are thought to increase the risk of females to chronic orofacial pain conditions including migraine, TMD, and trigeminal neuralgia (Sacco et al., 2012).

Neurophysiology of Stress. Stress affects all systems of the body including the nervous and digestive systems, which are a focus of my studies. One of the most important neuroendocrine pathways related to stress is the hypothalamus-pituitary-adrenal axis, also known as the HPA axis, and is a component of the sympathetic nervous system. Acute stress stimulates the paraventricular nucleus in the hypothalamus to produce corticotropin releasing hormone that causes cells of the pituitary gland to secrete adrenocorticotrophic hormone, which acts as a signal to the adrenal gland to stimulate release of epinephrine, norepinephrine, and the glucocorticoid cortisol (Stephens and Wand, 2012). The rapid increase of cortisol and other hormones in causes increased respiration and heart rate, diverts blood flow towards skeletal muscles, and affects the way an individual feels pain, which are all beneficial responses to quickly adapt to a threatening situation (Kozłowska et al., 2015). In addition, activation of the HPA axis is known to influence the function of the digestive system resulting in slowing of gastric emptying, increase in colonic motility, elevation in cytokine levels, and may cause temporal changes in the microbiota (Enck et al., 1989). Chronic stress, however, can cause maladaptive changes to the HPA axis that last long after the stressor is removed (Abdallah and Geha, 2017). This is due in part to the allosteric load on the HPA axis from sustained elevated levels of glucocorticoids and hormones (Silverman and Sternberg, 2012). Chronic exposure to stress can also result in changes in brain structure and function and importantly can mediate cellular changes in receptors, ion channels, and signaling pathways that lower the activation threshold of neurons to sensory stimuli (Mariotti, 2015). In addition, chronic stress is likely to lead to negative changes in the proper functioning of the digestive system that can promote development of stomach

ulcers, inflammation of the colon, colitis, increase risk of infections, and dysbiosis of the microbiota (Farzi et al., 2018; Rea et al., 2016). Chronic stress early in life is thought to exert a larger impact on the HPA axis and nervous system than chronic stress experienced in adults due to the stress occurring during the formative development of the brain and digestive system (Jensen et al., 2015; Winston and Sarna, 2016).

Early Life Stress. Childhood stress is a risk factor for many complex diseases later in life involving the nervous and digestive systems (Entringer et al., 2016). For example, early life stress has been linked to development of irritable bowel syndrome, chronic pain, migraine, and TMD later in life (Green et al., 2011; Tietjen and Peterlin, 2011). Unmanaged early life stress often leads to behavioral changes including poor sleep hygiene, poor dietary choices, and a more sedentary lifestyle, all of which exert a long-term negative impact on a child's health (Gavrieli et al., 2015). When children are affected by chronic stress there is an emotional and economic toll on the individual and their family that is likely to also impact behaviors at school and in society. There is much evidence to support the notion that childhood development is detrimentally affected by early life stress, which causes learning difficulties resulting in lower academic performance and may lead to maladaptive coping mechanisms like overeating or substance abuse later in life (Enoch, 2011; Pechtel and Pizzagalli, 2011). Children burdened by early life stress may have a maladaptive central nervous system that is characterized by a sensitized state of hypervigilance or hyperexcitability (Bath et al., 2016). In a minimal stress environment, children and adults are less likely to engage in destructive behaviors (Reebye, 2005). Additionally, like chronic stress in adults, early life stress is a risk factor for development of chronic inflammatory and pain conditions

including many that involve sensitization and activation of the trigeminal nerve such as migraine and TMD (Tietjen and Peterlin, 2011; Tietjen, 2016). Therefore, it is important to have a better understanding of the potential impact of early life stress to find ways to mitigate or eliminate the associated deleterious effects, and thus lower the psychosocial and economic burden of complex diseases and chronic pain conditions involving the trigeminal nerve.

Stress to a pregnant mother can also exert a profound effect on the developing nervous and digestive systems of children and can lead to ineffective coping mechanisms and health complications later in life. During gestation, a mother's stress can imprint on the fetus in utero, resulting in changes similar to changes occurring from stress during infancy (Zucchi et al., 2012). This imprinting is commonly referred to as epigenetic inheritance. With epigenetic inheritance, the offspring's genome remains unchanged since this type of inheritance does not involve mutations or changes in the DNA sequence. However, gene expression can be altered in a dynamic and reversible manner via activity of microRNAs, as well as histone modifications and DNA methylation that influence packaging of the DNA, and hence accessibility to particular genes (Vasilatou et al., 2013). Epigenetic factors help explain why differentiated cells remain specialized and why identical twins have different health outcomes based on their life style and environment. Exposure to prenatal stress can mediate changes in an offspring's epigenome to favor development of a hypersensitive HPA axis nervous system. However, the physical, emotional, and social environment of the individual after birth also plays a major role in their epigenetic signature and thus their risk of developing chronic pain conditions or a maladaptive nervous system. Previous studies have characterized the

negative effects in offspring of primary stressed mothers (Golubeva et al., 2015; Thayer et al., 2018) and more recently mothers exposed to secondary traumatic stress before mating, during gestation, and during nursing (Hawkins et al., 2018). Results from our laboratory provided evidence that secondary stress to the mother and early life stress to the offspring was sufficient to cause increased expression of pro-inflammatory signaling proteins in the trigeminal ganglion and spinal trigeminal nucleus associated with peripheral and central nervous system sensitization, key events in migraine pathology (Bernstein and Burstein, 2012; Nosedá and Burstein, 2013; Su and Yu, 2018).

Trigeminal Nerve and Migraine

The trigeminal nerve is the fifth cranial nerve and as the name implies, is comprised of three branches that provide sensory innervation of the major regions of the head and face. The three branches are designated as V1, V2, and V3, and peripheral neuronal fibers respond to mechanical, thermal, and chemical signals from the ophthalmic, maxillary, and mandibular regions, respectively (Figure 1). Trigeminal sensitization can be observed through mechanical stimulation of the regions of the face that are innervated by the respective branch (Garrett et al., 2012; Sessle, 2011). When an area of the face is presented with a potentially dangerous stimuli such as a sharp increase in temperature or mechanical force, neurons specialized to transmit pain signals, called nociceptors, with terminals in that region send signals via the trigeminal nerve, which then relays nociceptive signals to the spinal cord and ultimately to the brain for processing (Dubin and Patapoutian, 2010). The two main types of nociceptors, A delta fibers and C fibers, transmit signals at different velocities. A delta fibers are myelinated,

will transmit signals quickly, and will cause a sharp pain that will dissipate within seconds. C fibers, however, are unmyelinated, transmit signals slower, and are responsible for the dull pain that lingers after the acute pain signals from the A delta fibers terminate. Based on the strength of the stimulus, activation of trigeminal nociceptive neurons may result in a withdrawal response and other reflexive autonomic protective responses. Often people with a chronic pain condition will experience the phenomena of allodynia, which is defined as a painful response to a normally non-harmful stimulus such as brushing one's hair or wind blowing against one's face (Janig, 2011). This type of enhanced response, which is reported to occur in migraineurs, triggers a voluntary withdrawal reflex because of a lowered activation threshold of C fiber nociceptor neurons such that a typically non-painful stimulus is now perceived as painful. In addition, migraineurs are reported to have a hyperexcitable or hypervigilant nervous system that predisposes them to reported migraine triggers that include changing light patterns, strong or pungent odors, particular foods, and other types of sensory stimuli (Levy et al., 2009; Yan and Dussor, 2014). Furthermore, data from a recent genome-wide association study supported the notion that the underlying pathology of migraine is more closely correlated with irritable bowel syndrome than other types of neurological disorders (Goadsby et al., 2017; Wang et al., 2017).

Microbiome-Gut-Brain Axis

The microbiome-gut-brain axis is defined as the interaction and influence between the intestinal bacteria, their metabolites, the enteric nervous system, and the central nervous system (Galland, 2014). The tenth cranial nerve, also called the vagus nerve, is a

key component of the microbiome-gut-brain axis (Figure 2) and is implicated in intestinal permeability (Costantini et al., 2010). It is now appreciated that different strains of bacteria can have a direct influence on the function of the central, and likely peripheral, nervous systems via the synthesis of cytokines, unique metabolites, and neurotransmitters including gamma amino butyric acid (GABA) and serotonin (Galland, 2014). The microbiome-gut-brain axis is bidirectional since metabolites produced in the gut can influence specific regions in the brain that influences signaling from the brain to the periphery via transmission through spinal cord neurons and the vagus nerve (Bonaz et al., 2018). The direct connection between the gut and brain is not a new concept but rather is one that has not been well-studied at the cellular/molecular level in connection with human disease until more recently. The fact that diet is key to good physical, mental, and emotional health has been known since the times of Hippocrates. Sudden dietary changes are known to negatively impact the mood of individuals causing them to feel less energetic, which can perpetuate a vicious cycle of craving higher caloric foods with less nutrition. For example, eating a high sugar, low fiber diet can promote a depressed and irritable state characterized by inflammatory changes in the digestive and nervous systems such that the individual seeks more calorie dense foods (Rogers et al., 2016). Similarly, individuals suffering from depression or unmanaged anxiety are at risk for developing ulcers and irritable bowel syndrome (Gao, 2013; Hsu et al., 2015). Hence, there is much clinical evidence to support a strong association between the health of one's gut and brain that is key to maintaining and restoring homeostasis and preventing chronic disorders.

The development of a chronic pain state is dependent on many genetic and environmental factors that influence the normal functioning of the gut-brain axis and ultimately the health of the gut microbiota, which play a key role in controlling inflammatory processes within the intestines. Chronic inflammation of the intestines due to epithelial infiltration of lipopolysaccharides and other immune system stimulating factors causes the production of inflammatory cytokines that influence the local environment but can also be released systemically (Rubin et al., 2012). Given that migraine and irritable bowel syndrome are associated with systemic inflammation and endothelial/epithelial permeability, the fact that these two disorders are often comorbid should not be surprising (Camara-Lemarroy et al., 2016). Throughout the past decade, the gut microbiome has been recognized as having a larger impact on overall health than previously thought. The gut microbiome refers to the biotic and abiotic factors within the intestines that include the bacteria, their genes, and all metabolites and molecules found within the intestines. The gut microbiota, which refers only to the bacteria in the intestines, serves several important functions that affect overall health and are much easier and cost effective to identify than the entire microbiome. Gut bacteria stimulate the immune system at an early age, leading to increased epithelial differentiation and stronger intestinal integrity (Peck et al., 2017). In addition, they can aid in digestion and extraction of nutrients from foods that mammalian enzymes cannot degrade such as plant fibers (Krajmalnik-Brown et al., 2012). Additionally, they can synthesize molecules that mammals cannot synthesize but depend on for overall digestive and nervous system health including short chain fatty acids, and other neuroregulatory molecules, like GABA and serotonin (Ohira et al., 2017; Yano et al., 2015). The normal production of these

metabolites and in particular, short chain fatty acids, is required to maintain a balance between the immune system and microbiota to ensure a healthy epithelium (Peng et al., 2009).

Dysbiosis occurs when there is an imbalance in the microbiota. Gut dysbiosis is almost always found in association with chronic inflammatory diseases such as irritable bowel syndrome, rheumatoid arthritis, and diabetes; and it is often reported in mental health conditions like autism spectrum disorder, major depressive disorder, and chronic stress induced anxiety (Koh and Kim, 2017; Rogers et al., 2016). Weakened intestinal epithelial integrity, characterized by a decrease in adherens junctions, tight junctions, and desmosomes, is often seen in conjunction with dysbiosis and inflammatory diseases, allowing infiltration of metabolites from the lumen into the blood stream, further promoting inflammation (Luissint et al., 2016). Increased intestinal permeability can allow potentially noxious and foreign particles such as proteins, partially digested food, and parts of bacteria through the epithelial barrier where an immune response will ensue to eliminate the foreign antigen (Luissint et al., 2016). Dysbiosis and increased intestinal permeability mediated by a poor diet and unmanaged anxiety and depression can lead to a cyclic inflammatory state within the intestine and systemic inflammation, which is a risk factor for development of chronic pain.

Therapeutic Approaches and Enriched Chicken Bone Broth

Throughout history, humans have been cooking animal bones and tissues to make nutritious broths to maintain and restore their health. Even today, in most cultures, when an individual has a respiratory infection it is recommended to eat chicken soup to

promote healthy physiological mechanisms. In support of this notion, results from *in vitro* studies have provided evidence that chicken broth inhibits neutrophil chemotaxis and increase nasal mucus velocity, which would help with the symptoms of a respiratory infection (Rennard et al., 2000; Saketkhoo et al., 1978). Interestingly, despite the widespread consumption of chicken soup and bone broths, there are few human clinical studies that actually document the potential benefits at the cellular and molecular levels. However, findings from our laboratory have recently shown that daily inclusion of an enriched chicken bone broth as a dietary supplement in Sprague-Dawley rats can reduce nociception in a jaw injury model and exhibits anti-inflammatory properties (Hawkins and Durham, 2018). Based on the results of this study, I will be investigating the possible therapeutic benefits of this enriched chicken bone broth on both nociception due to early life stress and its effects as a modulator of the gut microbiota.

Hypothesis and Goals

My central hypothesis is that changes mediated by early life stress, such as lowering of the activation threshold of trigeminal neurons and altering the gut microbiota, will be modulated by dietary inclusion of enriched chicken bone broth. To test my hypothesis, I plan to investigate the effect of early life stress on the trigeminal system to promote a persistent sensitized state of nociceptors in an acute model of migraine and correlate changes in the nervous system with changes in the gut microbiota using next-generation sequencing. Furthermore, I will test whether dietary inclusion of an enriched chicken bone broth can inhibit nociception and alter the gut microbiota. The acute migraine model is based on a recently published study from our laboratory (Hawkins et

al., 2017) in which sensitized male Sprague-Dawley rats exposed to a pungent odor, known to be a migraine trigger in humans, caused activation of trigeminal neurons and enhanced nociception to mechanical stimulation. Although only male animals were used in that prior investigation, I have chosen to include both male and female animals in my study since migraine is more prevalent in females, and I expect to see basal gender differences in nociceptive responses and gut microbiota.

Knowing that early life stress can mediate pro-inflammatory and pro-nociceptive cellular changes in both the trigeminal ganglion and spinal trigeminal nucleus (Hawkins et al., 2018), I predict that animals exposed to early life stress will have a greater level of basal mechanical sensitivity and a lower threshold of nociception, which is likely to be more pronounced in females. Given the gut-brain axis connection, I predict that both female and male offspring of stressed mothers will exhibit a shift in their gut microbiota that will be more severe in female animals. Furthermore, I predict that early intervention via dietary inclusion of an enriched chicken bone broth will protect the animals from developing a sensitized state characteristic of migraine and other orofacial pain conditions.

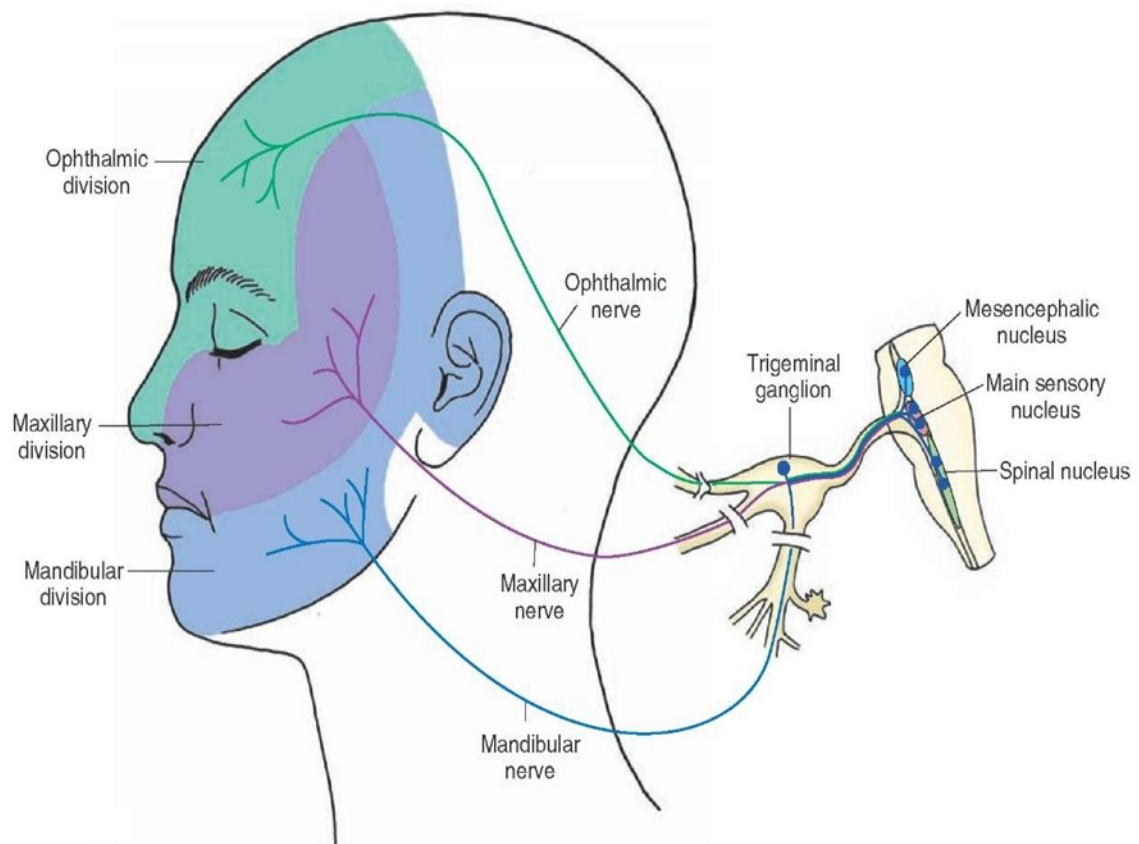


Figure 1. Anatomy of the trigeminal nerve. This illustration shows the dermatomes in the head and face innervated by the three branches of the trigeminal nerve including the V1 (Ophthalmic), V2 (Maxillary), and V3 (Mandibular). Sensory information travels from the peripheral tissues through the trigeminal ganglion and terminate in the brainstem. Retrieved from <http://what-when-how.com/neuroscience/the-cranial-nerves-organization-of-the-central-nervous-system-part-3>.

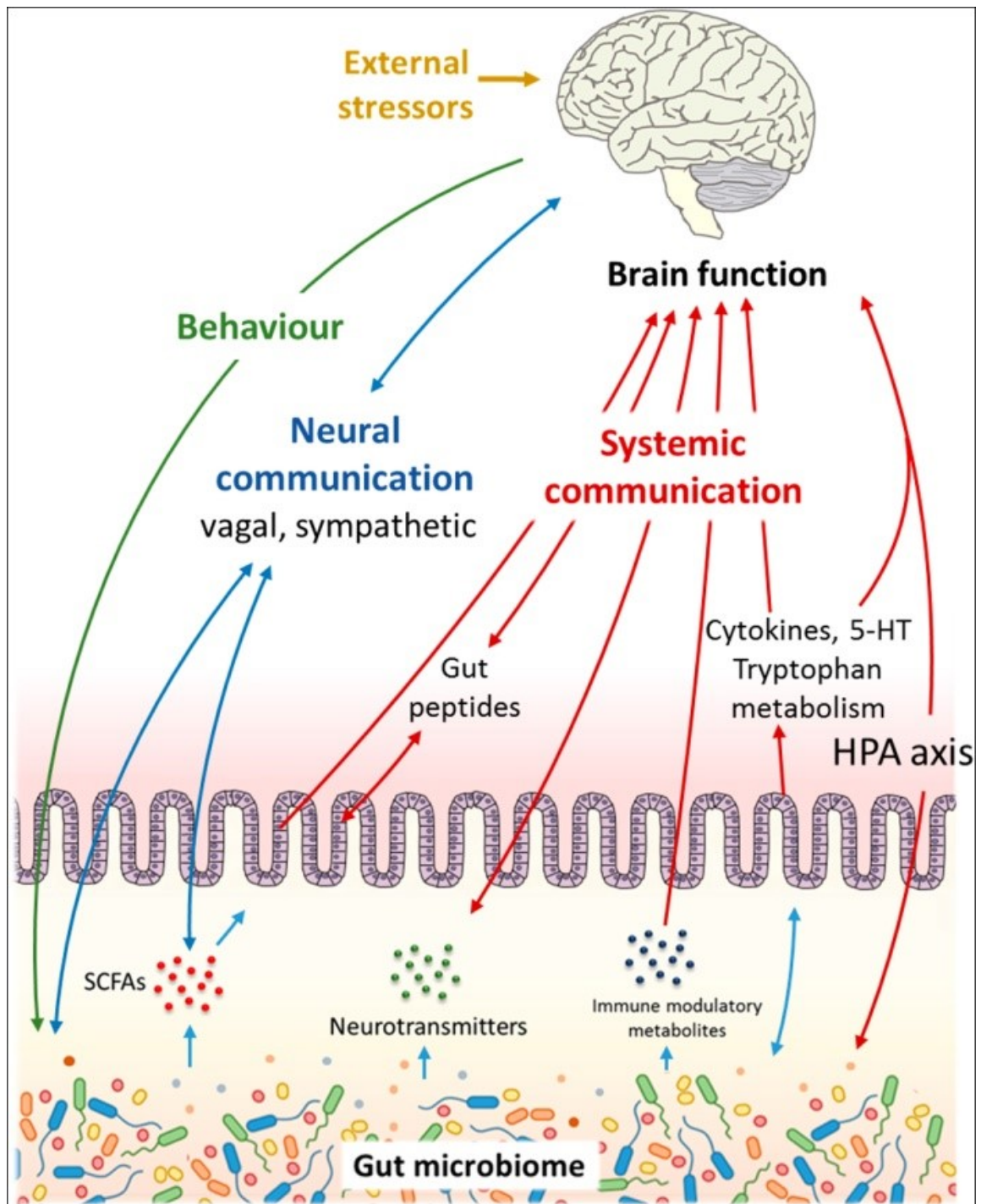


Figure 2. Summary of normal physiological and cellular interactions between the central nervous system and gut microbiota. Adapted from Rogers et al., 2016.

METHODS

Animals and Experimental Model

All Sprague-Dawley rats used in this study were purchased from Missouri State University's Central Animal Management (internal breeding colonies). The rats were housed in clean plastic cages (VRW, West Chester, PA) with bedding and Crink-I'Nest™ (The Andersons Inc., Maumee, OH) and had unrestricted access to water or broth and food. During pregnancy and nursing, mothers received Formulab Diet (product code 5008, LabDiet, St. Louis, MO), while males and offspring were fed Laboratory Rodent Diet (product code 5001). The vivarium rooms were on a 12-hour light/dark cycle and were held between 20-24°C and 30-70% humidity. All animal care and protocols were approved by Missouri State University's Institutional Animal Care and Use Committee (IACUC ID: 17-010.0). Additionally, an effort was made to reduce the number of animals in this study and to limit their suffering.

The experimental model of early life stress was based on a recently published study from our laboratory (Hawkins et al., 2018). Briefly, non-breeding male rats (Day >70) were subjected to forced swimming in a Morris water maze to induce primary traumatic stress. These animals were designated as sender animals. The male swimmers were briefly placed on a slightly submerged, clear plastic platform, then placed in the water in front of one of the spatial cues (Figure 3A). The rat would swim until it pulled itself onto the platform or for no more than three minutes, at which point the rat would be removed and allowed to rest in a cage with paper towels for two minutes. This stressful event was repeated three additional times in succession, then the animal was returned to

its cage and placed between an age-matched male and female rat, which were designated as receiver animals. This procedure was repeated every day for a week at three specific times that included the time of breeding, pregnancy, and after weaning as detailed in Table 1.

A male and a female rat that were not subjected to swimming were selected to be bred and were housed in separate cages on either side of the swimmer rat at all times (Figure 3B) except for when the two rats were cohoused in order to copulate. As shown in the experimental design in Table 1, the swimmer rat was exposed to Morris water maze swimming for a week before the mating of the parents, a week after the parents were allowed to copulate, usually falling halfway between the first and second week, and a week following weaning of the offspring (postnatal days 21-28). In summary, the fathers were exposed to secondary stress before they mated with the mothers; the mothers were exposed to secondary stress before copulation and during gestation; and the offspring were exposed to secondary stress after they were weaned. A naïve control group was used in which the same timeline was followed, but there was no exposure to a primary traumatic stressed animal. From day 21 onwards, offspring with early life stress were provided either water (control), 1% mass/volume AAC1 enriched chicken bone broth, or 0.66% mass/volume Granny 4 (G4) chicken bone broth diluted in their drinking water. Both bone broth preparations were supplied by International Dehydrated Foods (Monet, MO) and were utilized in a prior study from our laboratory involving trigeminal nociceptor activation (Hawkins and Durham, 2018).

Nociception Testing

The basal nocifensive response to mechanical stimulation of the cutaneous tissue over the temporalis (V1) or masseter (V3) was performed as described previously (Garrett et al., 2012). All offspring (n = 101) were acclimated at seven weeks old to a Durham Animal Holder (UGO Basile, Italy), which provides a novel method for securing the animal with minimal stress (Figure 4A and 4B). Animals were restrained within the device for 5 minutes while the fur around the head and face was brushed with a von-Frey filament (North Coast Medical, Inc., Gilroy, CA; 60, 100, 180 grams) to familiarize the rats with the filament and minimize false responses due to unexpected fur stimulation. This was done for three consecutive days, after which the animals were allowed to rest for at least two days before the start of the experimental procedure. On the day of testing, a series of calibrated von-Frey filaments ranging from 60g-180g force (Figure 4C) were applied five times to the cutaneous areas over the left and right temporalis muscle (ophthalmic; V1) and masseter muscle (mandibular; V3) five times. The number of head withdrawal responses out of 5 applications was recorded for each side for the V1 and V3 regions, and an average of both sides was used to report data. The 60g filament in the V1 region and the 100g filament in the V3 region were the filaments chosen to report because at these weights the average response was less than 1 out of 5 applications. A female scientist in our laboratory conducted the nociception testing in collaboration with myself or another scientist. The scientist performing the testing was blinded to the experimental condition of the animal. Data are reported as the average number of responses out of 5 applications \pm the standard error of the mean (SEM) of the filament of interest. Each experimental condition was repeated in a minimum of 4 animals.

To determine if early life stress could function as a risk factor to lower the activation threshold of trigeminal neurons to mechanical stimulation, some animals ($n = 56$) were exposed to a pungent odor known to cause activation of sensitized trigeminal neurons in an animal model of acute migraine (Hawkins et al., 2017). Animals were exposed for 10 minutes to a pungent odor from a California bay leaf oil extract (CBL) by placing 20 μL of the oil on a cotton swab and placing the animal in a chamber with an oxygen flow of 2 L/min. The cotton swab was positioned so that the animal could not come into direct contact with the oil but would be subjected to the oil's volatile compounds including umbellulone (Nassini et al., 2012). Mechanical nociception testing was performed prior to CBL exposure to determine basal levels and then again at 2, 24, and 48 hours post exposure. Animals were sacrificed after all testing was complete by CO_2 asphyxiation and decapitation. After all behavioral data was generated, eight outliers were removed from the study. Outliers were defined as any animal that responded greater than or less than 2 standard deviations from the mean at least once in both the V1 and V3 regions at any of the time points. Behavioral data were analyzed using non-parametric tests on SPSS 24 software (IBM, North Castle, NY). Wilcoxon rank sum tests were performed to determine if two measurements within the same group were statistically different from one another and Mann-Whitney U tests were performed to determine if two measurements from the same time point across two groups were statistically different. Two measurements were considered significantly different when $p < 0.05$.

Microbiota Analysis

To obtain fecal and cecum samples for microbiota analysis, some animals (n = 45) were sacrificed by CO₂ asphyxiation and decapitation immediately after basal nocifensive behavioral testing. A sample of the contents of the cecum and the most distal fecal samples were collected directly from the animal at the time of dissection and were stored at -20° C.

DNA from 100 mg of fecal and cecum samples was extracted using the QIAamp® Fast DNA Stool Mini Kit, from Qiagen (Catalogue ID: 51604, Germantown, MD) according to the manufacturer's instructions. DNA concentrations were measured spectrophotometrically and samples from the same treatment group and sex were pooled evenly. Outliers were identified based on mechanical nociception testing that were more than or less than 1.5 standard deviations away from the mean for both the V1 and V3 tests. These four outliers' fecal and cecum samples were not included in the pooling of DNA for the microbiota analysis; however, after the completion of the study, they were added back to the behavioral data since none of them qualified as outliers based on my definition of outliers stated in the "nociception testing and statistics" section. Pooled samples [around 20 µL of 20 ng/µL DNA solution in Qiagen's Buffer ATE (10mM Tris-Cl pH 8.3, 0.1mM EDTA, 0.04% NaN₃)] were sent to MR DNA [Molecular Research LP, Shallowater, TX, USA (www.mrdnalab.com)] where PCR was performed using the HotStarTaq Plus Master Mix Kit from Qiagen with primers that corresponded to the V4 variable region of the 16S rRNA gene 515/806 (Caporaso et al., 2011). PCR was initiated by heating the sample to 94°C for 3 minutes followed by thirty cycles of denaturing at 94°C for 30 seconds, annealing at 53°C for 40 seconds, and elongation at 72°C for 60

seconds. A final elongation step at 72°C for 5 minutes was performed. Sequencing was also performed at MR DNA on an Ion Torrent Personal Genome Machine following the manufacturer's guidelines.

Bacterial 16S rRNA gene sequences obtained from different treatments were filtered for initial quality control by MR DNA by removing DNA sequences containing more than one inexact match with the unique barcode identifier, read length <150 bp, containing > 6 homopolymer bases, and sequences with unidentified bases (Ns). After initial sequencing, chimeric sequences were removed using Ribosomal Database Project Release 11 (RDP11: <http://rdp.cme.msu.edu>). Then, OTUs were defined by clustering at 1% divergence and were identified using nucleotide-nucleotide Basic Local Alignment Search Tool (BLASTn) against a database derived from National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) and RDP11. Finally, "counts" and "percentage" files from each taxonomic level were generated. The "percentage" files were utilized to report the relative percentage of bacteria at the family and genus levels. No statistical analysis could be performed on the sequence data, since the 16 samples that were sent to MR DNA consisted of pooled DNA samples.



A



Father

Swimmer

Mother

B

Figure 3. Induction of early life stress. A) An image of the Morris water maze used in my study with the clear, underwater platform and colored shapes as visual cues. Only the non-breeding male was exposed to the Morris water maze. B) The non-breeding male swimmer (primary stressed, sender animal) was housed beside the breeding father and mother (secondary stressed, receiver animals) at all times.

Table 1. Timeline for early life stress model. The type of activity and temporal sequence is provided for each of the animal subsets utilized in my study.

Week	Activities			
	1° Stressed Male (Swimmer)	2° Stressed Male (Father)	2° Stressed Female (Mother)	Offspring
0	Swimming	Sitting	Sitting	
1	Sitting	Breeding	Breeding/Gestating	Gestating
2	Swimming	Sitting	Gestating	Gestating
3	Sitting	Sitting	Gestating	Gestating
4	Sitting	Sitting	Birth/Nursing	Born/Nursing
5	Sitting	Sitting	Nursing	Nursing
6	Sitting	Sitting	Nursing	Nursing
7	Swimming	Sitting	Sitting	Weaned ¹
8	Sacrificed	Sacrificed	Sacrificed	Sitting
9				Sitting
10				Sitting
11				Acclimation
12				Testing
13				Sacrificed

¹Offspring were removed from their mothers at postnatal day 21 and placed into cages with 1-3 siblings of the same sex and were provided unlimited access to either water (control) or a broth.

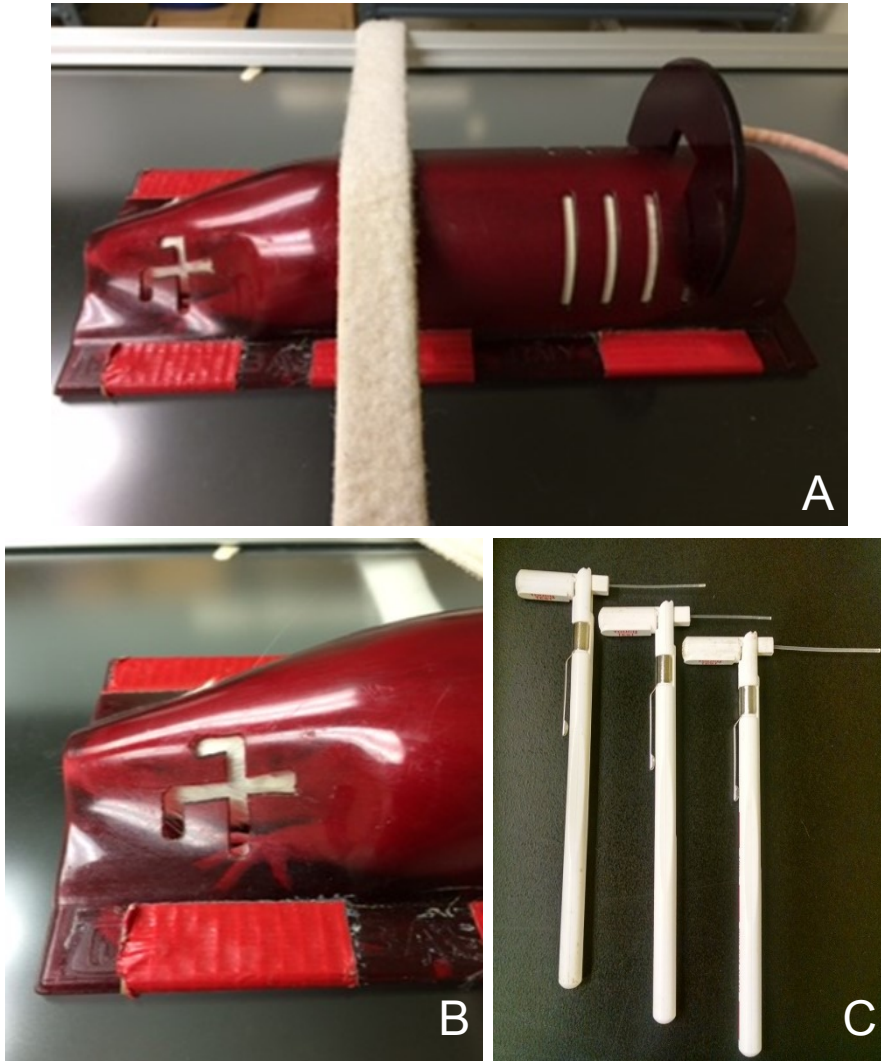


Figure 4. Experimental design for behavioral testing of mechanical sensitivity to nocifensive stimulation. A) Sprague-Dawley rat restrained in Durham Animal Holder. B) An image showing the cut out areas allowing access to regions of the face. C) From left to right: von-Frey filaments of 60g, 100g, and 180g. The von Frey filaments of interest were a 60g filament for the V1 region (temporalis) and a 100g filament for the V3 region (masseter). The 180g filament was used as a positive control if needed.

RESULTS

Testing Nociception

Behavioral studies in the orofacial region were performed to determine if rats with early life stress would produce nocifensive responses commonly seen in patients with migraine or TMD. Animals were defined to be sensitized if they responded to the filament of interest with a nocifensive head withdrawal on average of more than 2.5 times out of 5 independent stimulations. The 60g von-Frey filament was determined to be the filament of interest over the ophthalmic region (V1) because the naïve animals responded to the filament less than 1 time out of 5 on average, whereas the next largest filament caused the majority of naïve animals to respond to every stimulation. The 100g filament was determined to be the filament of interest for the V3 region for the same reasons previously mentioned. A 180g filament was used as a positive control as necessary. The average number of nocifensive head withdrawals from naïve animals and animals exposed to early life stress (ELS) with water, enriched chicken bone broth (AAC1), or a homemade broth (G4) for both the V1 and V3 regions can be seen in Figures 5 and 6, respectively. There was no significant difference between the basal male and female V1 and male V3 readings of animals under any condition. However, basal readings for females at the V3 region show females exposed to ELS and water were significantly higher than both the readings of the naïve females and the females exposed to ELS that were given AAC1 ($p=0.035$; $p=0.012$).

Figures 7 through 12 only show results from animals that were exposed to the noxious stimulant, California bay leaf oil extract (CBL). After exposure to CBL, two

animals from the ELS female with water condition became so severe in the V3 region that they became unresponsive to the filament of interest (100g) as well as the next highest filament (180g) that was used as a positive control for the naïve animals. The two animals with this characteristic were given a score of 6 rather than a 5 to indicate their severe behavioral reaction caused by excessive nociceptive signaling. Figures 7A and 7B show the results from a comparison of the basal readings and readings two hours after exposure to CBL of naïve males exposed to ELS with water. In both the V1 and V3 regions, the two conditions were not statistically significant at the basal time point, but two hours after exposure to CBL, the males with ELS increased significantly from their basal timepoints ($p=0.033$; $p=0.012$) and were statistically significant from the naïve males at the same two hour time point ($p=0.011$; $p=0.001$). Figure 8 displays results from the same conditions and timepoints as Figure 7, but in females. In the V1 region (Figure 8A), the level of sensitization significantly increased from basal to two hours in females with ELS ($p=0.037$) and females with ELS were significantly more sensitized than naïve females in the V3 region (Figure 8B) two hours after exposure to CBL ($p=0.036$). Overall, ELS promotes sensitization of the trigeminal nociceptive neurons that lowers the activation threshold to the CBL pungent odor in both male and female animals.

Dietary inclusion of AAC1 in animals exposed to ELS was investigated to see if the enriched chicken bone broth could mitigate sensitization seen in animals who were provided water and were exposed to ELS (Figure 9 and 10). V1 sensitivity slightly decreased, but not significantly, in males with ELS and AAC1 when compared to ELS and water (Figure 9A), while V3 sensitivity decreased significantly as seen in Figure 9B ($p=0.024$). Results seen in Figures 10A and 10B provide evidence that animals with

AAC1 exhibited less sensitization two hours after exposure to CBL in the V1 and V3 regions, respectively, though the changes did not reach significance. The average basal reading reported in Figure 10B shows that both the naïve and the ELS with water groups were significantly more sensitive than the AAC1 group ($p=0.034$; $p=0.014$).

Additionally, the AAC1 group exhibited a statistically significant increase in sensitization from basal to the two hour time point in the V3 region in Figure 10B ($p=0.039$), though this significant increase was only to 1.1 reactions out of 5, which was in line with the naïve group and was not considered a sensitized condition.

Results in Figures 11 and 12 depict nociception readings at two additional timepoints: one day and two days after CBL exposure. In one round of my study, I did not continue nociception testing past the two hour time point and did not investigate the effects of AAC1. Thus, the sampling size for the “Naïve” and “Stressed” groups are smaller for the 24 hour and the 48 hour time points. In males, there was no significant difference in orofacial sensitization between any condition at the 24 hour and the 48 hour time points (Figure 11A and 11B). As seen in Figure 12, the female animals receiving AAC1 have significantly lower sensitization than females with ELS and water in the V1 region at 48 hours ($p=0.009$) and the V3 region at 24 hours after exposure to CBL ($p=0.016$).

Gut Microbiota Profiles

Gut microbiota results were evaluated at the phylum, family, and genus level. All samples from a condition were pooled and the sample size for all 16 conditions is one. Therefore, no statistical analyses were performed on these data. Firmicutes and

Bacteroidetes were the two most prevalent phyla in the cecum and the fecal samples. At the phylum level, all fecal samples reported higher abundance (3.4%) of Proteobacteria, the next highest abundant phyla, than their cecum counterparts by 2% on average (data not shown). ELS and AAC1 chicken broth treatments did not greatly alter the relative abundance of Firmicutes, Bacteroidetes, or Proteobacteria across gender or across sample types. One general trend that was found was that Firmicutes are more prevalent in the cecum than they are in the colon.

Family abundance was then calculated for all samples and is represented in pie charts (Figure 13-16). While there are observable changes from the naïve animal in all of the other conditions, most are not conserved across sex or sample type. For example, Clostridiaceae, one of the most abundant families in the cecum and colon, is increased by 3.4% in the colon of males with ELS and AAC1 broth when compared to males with ELS alone (Figure 13), but is decreased in the colon of females by 13.7% (Figure 14). Conversely, Clostridiaceae is decreased by 5.8% in the cecum of males with ELS and AAC1 in Figure 15 and Figure 16 shows that it increased by 2% in females when compared to ELS alone.

Relative percent abundance of the top 15 most abundant genera for each sample type (fecal and cecum) are listed in tables 2-5. The genera *Oscillospira* and *Coprococcus* were increased in animals with ELS compared to navies, while there was a trend towards decreased *Lachnoclostridium* and *Pseudoflavonifactor* genera in animals with ELS. Abundance of *Robinsoniella* was noticeably diminished in the cecum and fecal samples from females with ELS, while not having a difference in males. The males and females

with AAC1 or G4 both had an increase of *Ruminococcus* in their fecal samples but not their cecum samples when compared to the animals with ELS alone.

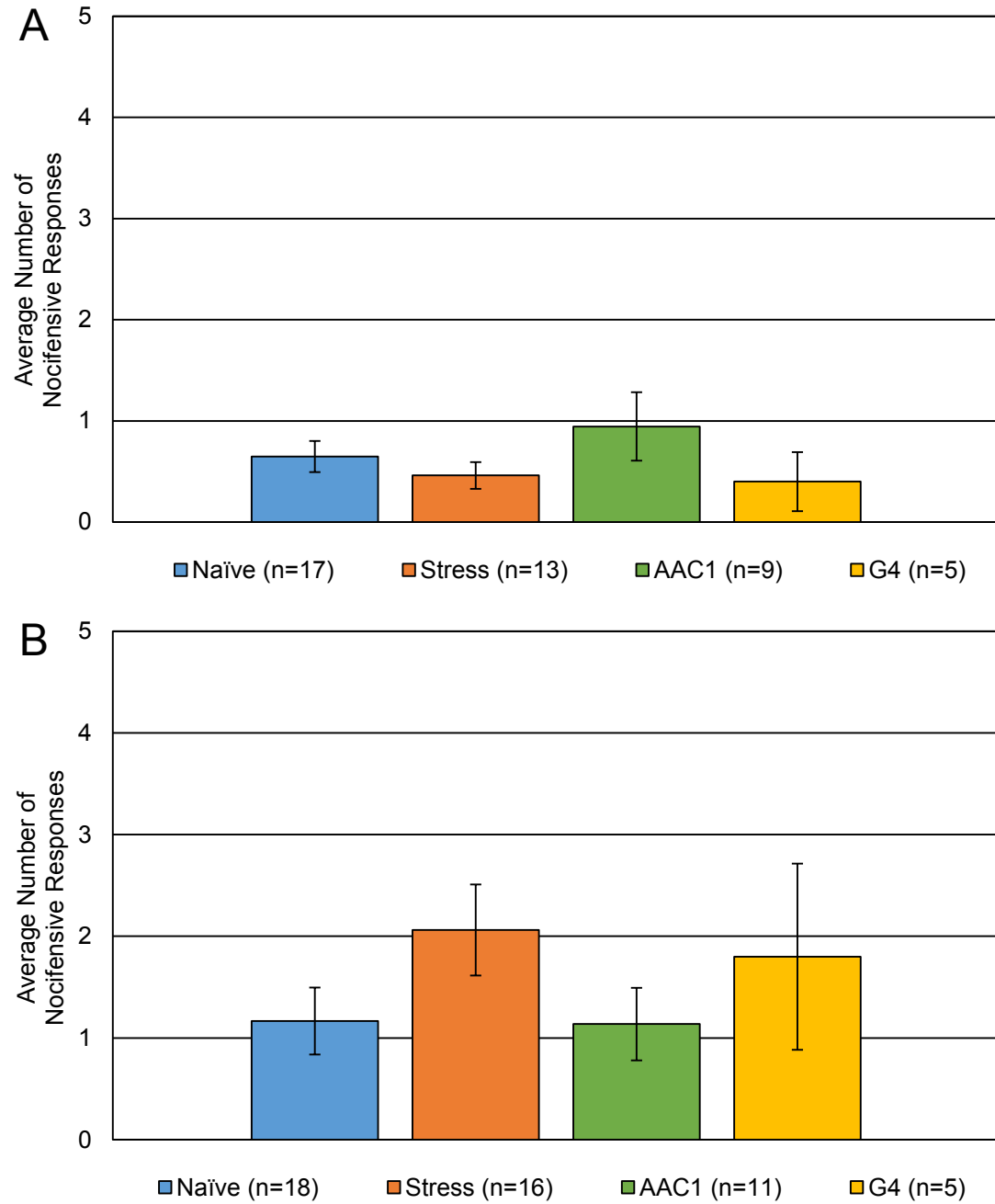


Figure 5. Early life stress does not cause V1 sensitization in male or female offspring. Results are shown for unstimulated naïve animals, animals exposed to secondary stress, or animals exposed to secondary stress and receiving dietary supplementation of enriched chicken bone broth (AAC1) or a homemade chicken broth (G4). 5A is a graph for male offspring and 5B is a graph for female offspring. The error bars are \pm SEM.

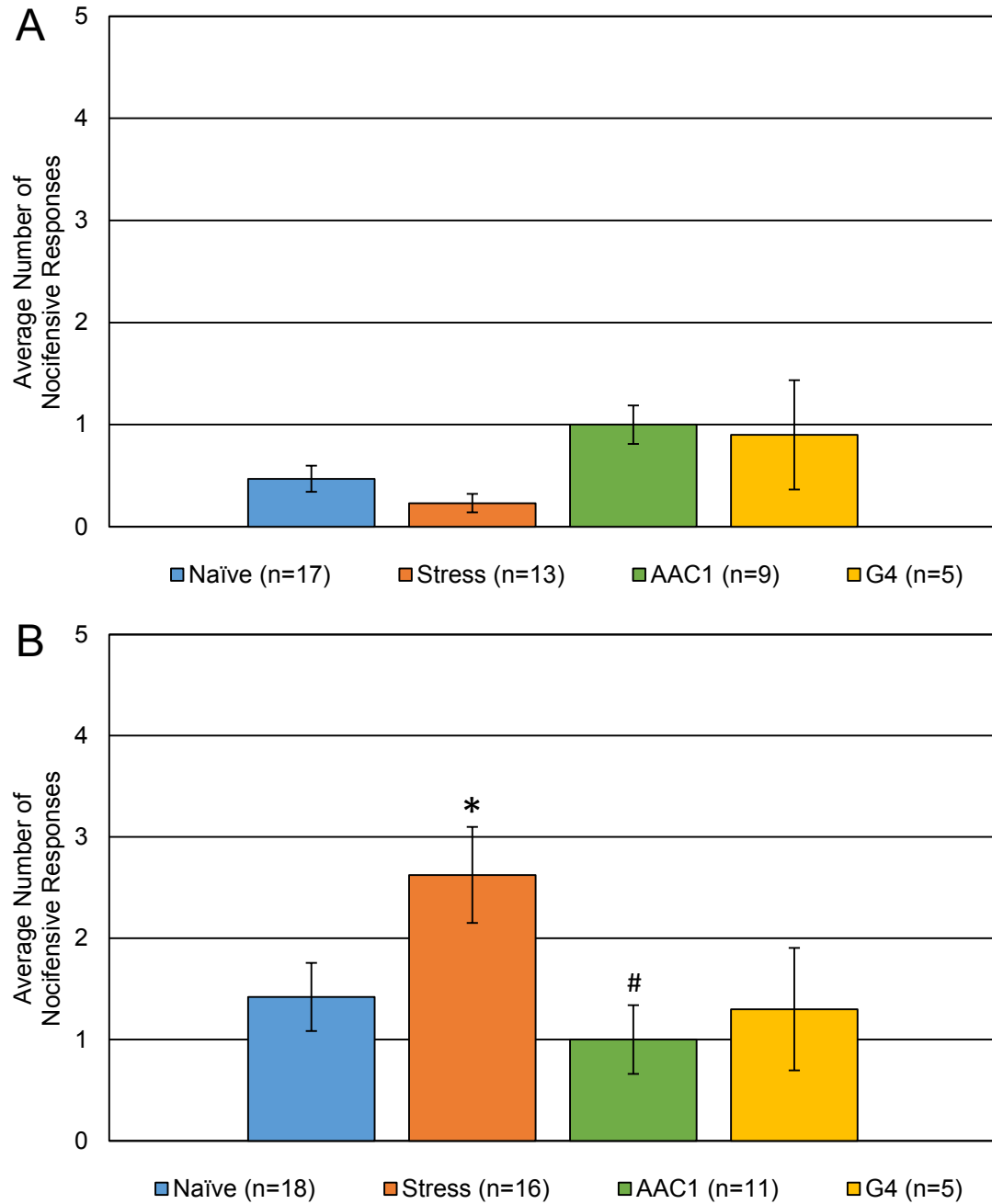


Figure 6. Early life stress causes V3 sensitization in female offspring but not male offspring. Results are shown for unstimulated naïve animals, animals exposed to secondary stress, or animals exposed to secondary stress and receiving dietary supplementation of enriched chicken bone broth (AAC1) or a homemade chicken broth (G4). 6A is a graph for male offspring and 6B is a graph for female offspring. The error bars are \pm SEM. A significant increase from the naïve animals is indicated by an asterisk, while a significant decrease from stressed animals is indicated by a pound sign.

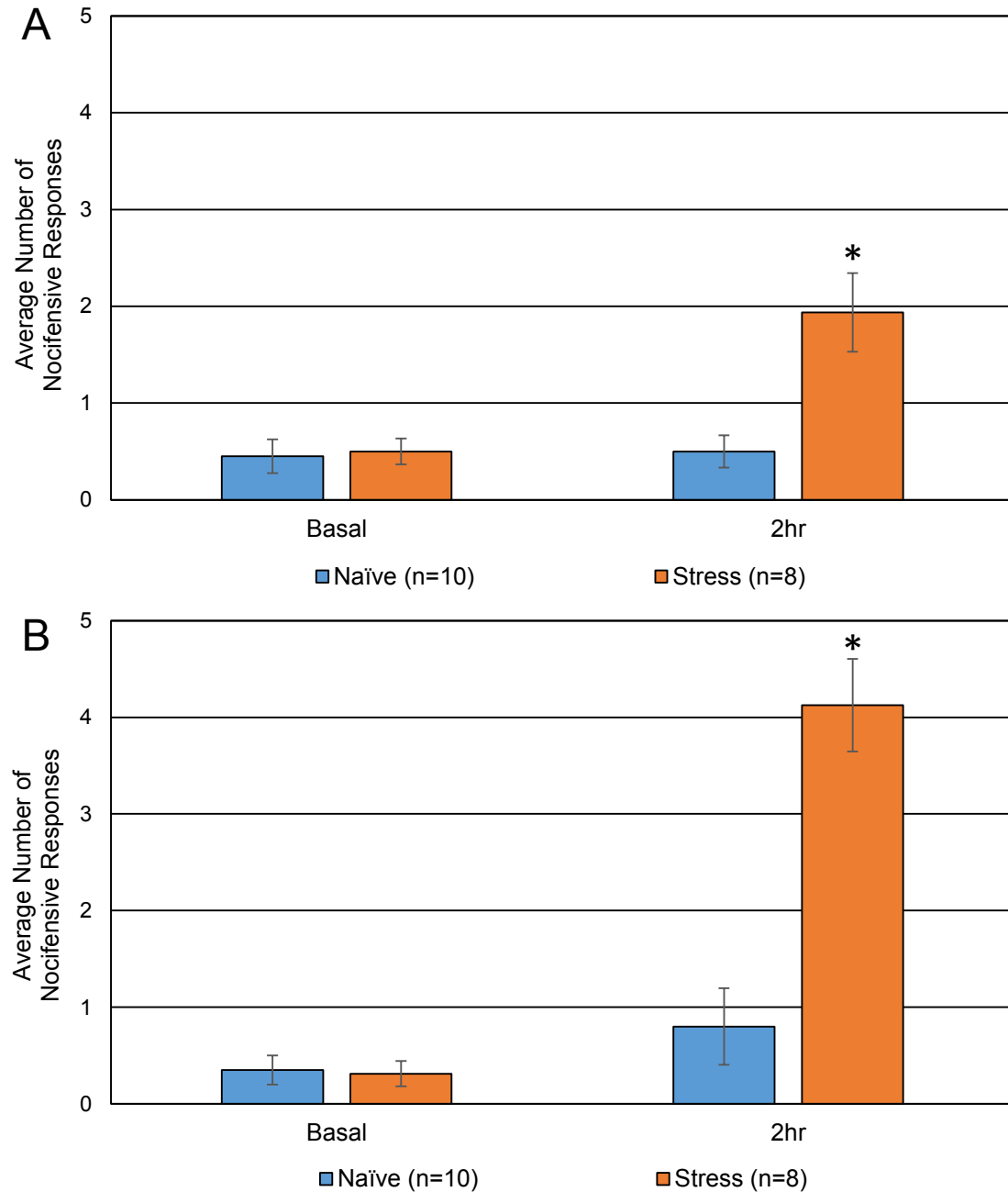


Figure 7. Exposure to migraine trigger V1 and V3 activation in male offspring exposed to early life stress. Basal measurements were taken before exposure to the bay leaf extract and 2hr measurements were taken two hours after exposure. 7A shows the level of V1 sensitization and 7B shows V3 sensitization. The error bars are \pm SEM. A significant increase from the naïve animals is indicated by an asterisk.

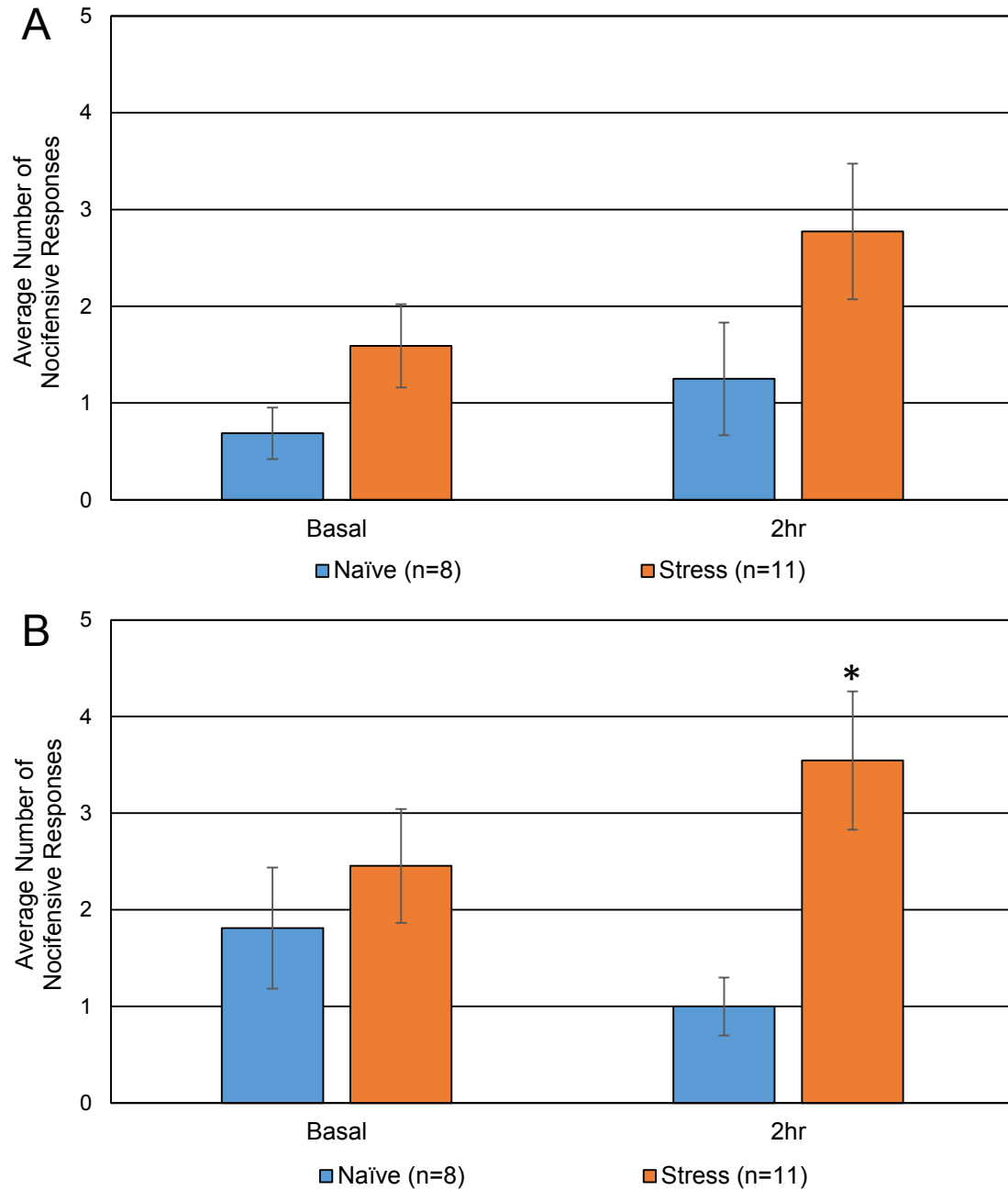


Figure 8. Exposure to migraine trigger V3 but not V1 activation in female offspring exposed to early life stress. Basal measurements were taken before exposure to the bay leaf extract and 2hr measurements were taken two hours after exposure. 8A shows the level of V1 sensitization and 8B shows V3 sensitization. The error bars are \pm SEM. A significant increase from the naïve animals is indicated by an asterisk.

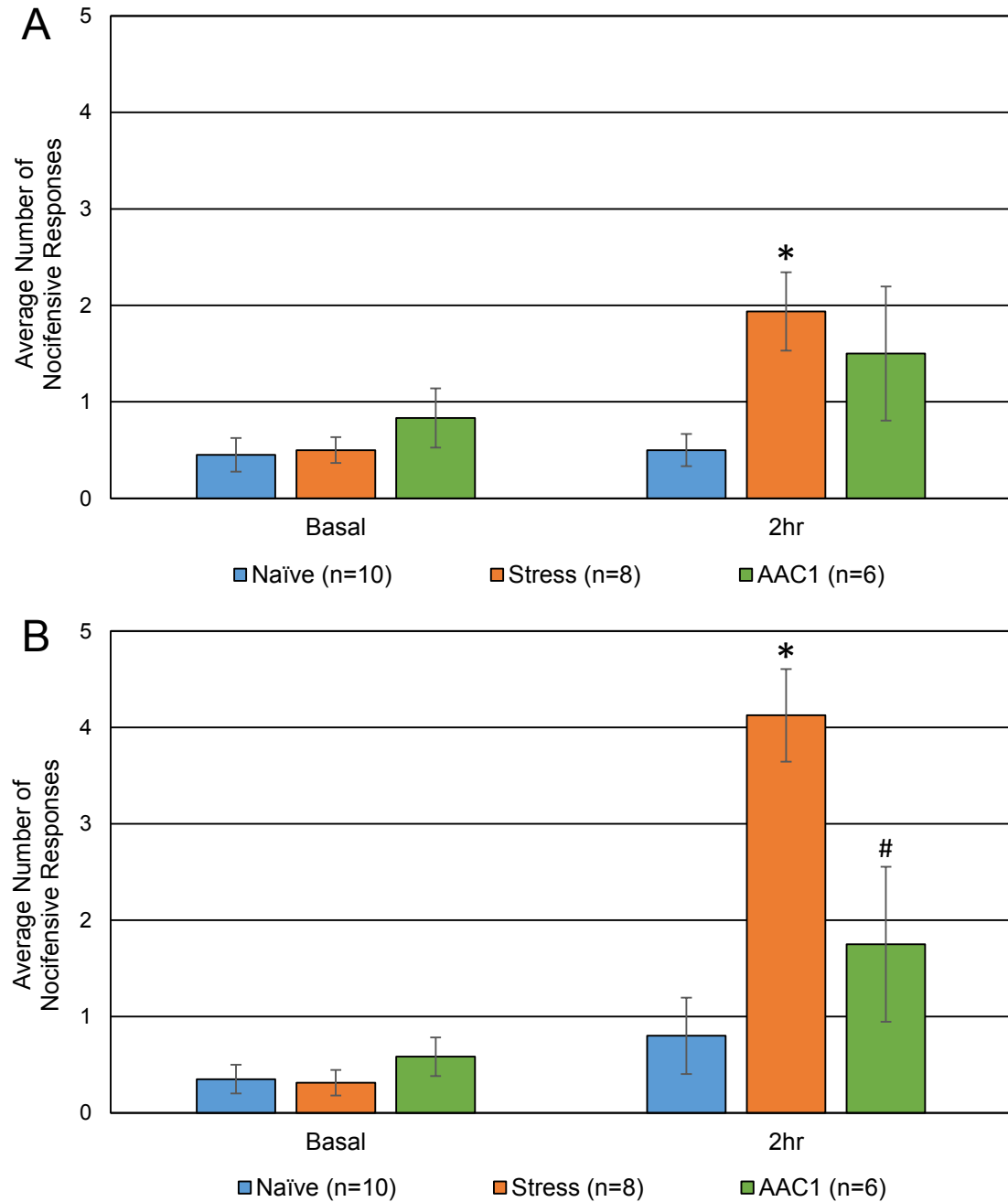


Figure 9. Dietary supplementation with enriched chicken bone broth (AAC1) inhibits V3 but not V1 nociceptive responses induced by migraine trigger in male offspring exposed to early life stress. Basal measurements were taken before exposure to bay leaf extract and 2hr measurements were taken two hours after exposure. 9A shows V1 sensitization and 9B shows V3 sensitization. The error bars are \pm SEM. A significant increase from the naïve animals is indicated by an asterisk while a significant decrease from stressed animals is indicated by a pound sign.

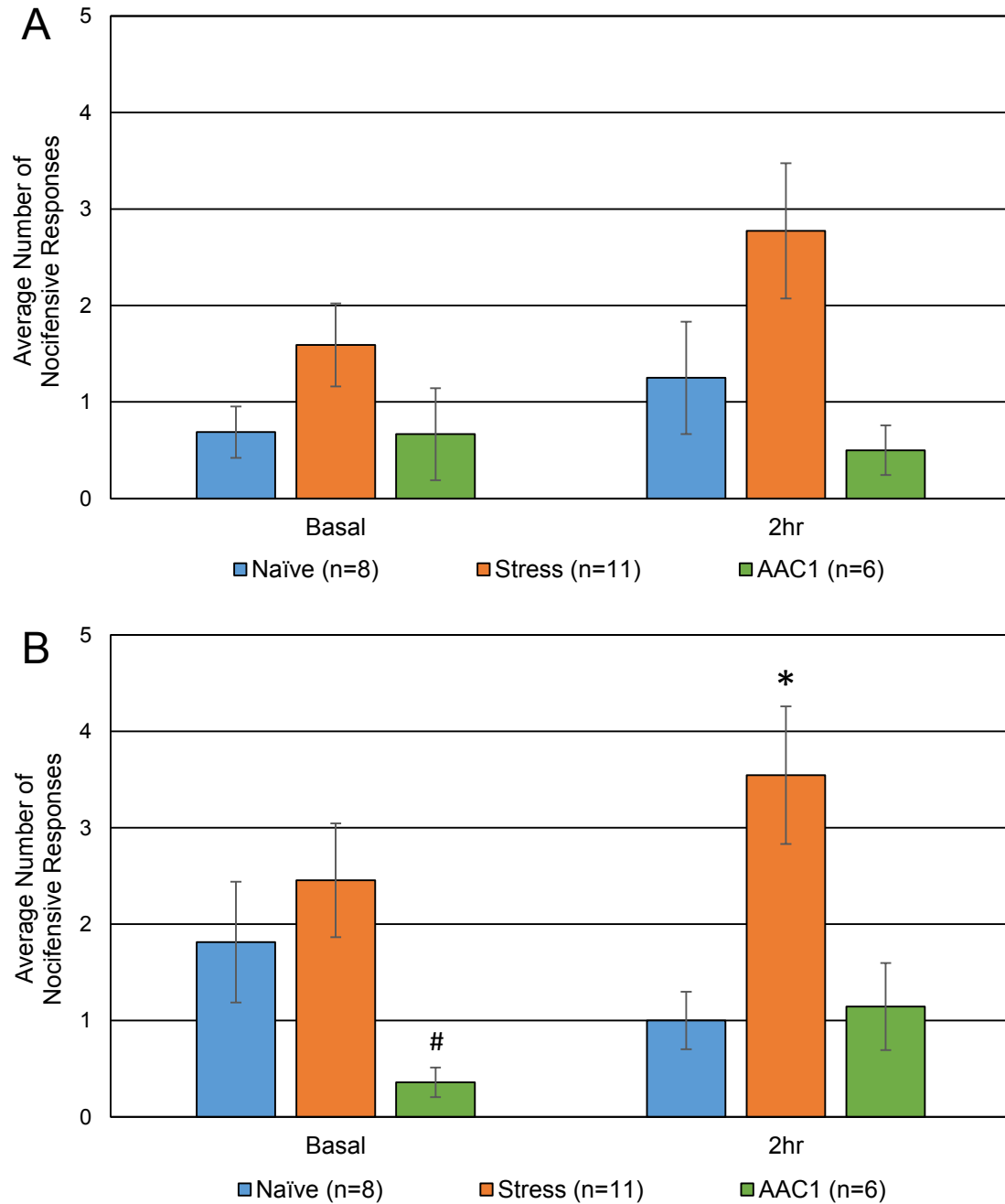


Figure 10. Dietary supplementation with enriched chicken bone broth (AAC1) inhibits V1 and V3 nociceptive responses induced by migraine trigger in female offspring exposed to early life stress. Basal measurements were taken before exposure to bay leaf extract and 2hr measurements were taken two hours after exposure. 10A shows V1 level of sensitization and 10B shows V3 sensitization. The error bars are \pm SEM. A significant increase from the naïve animals is indicated by an asterisk while a significant decrease from stressed animals is indicated by a pound sign.

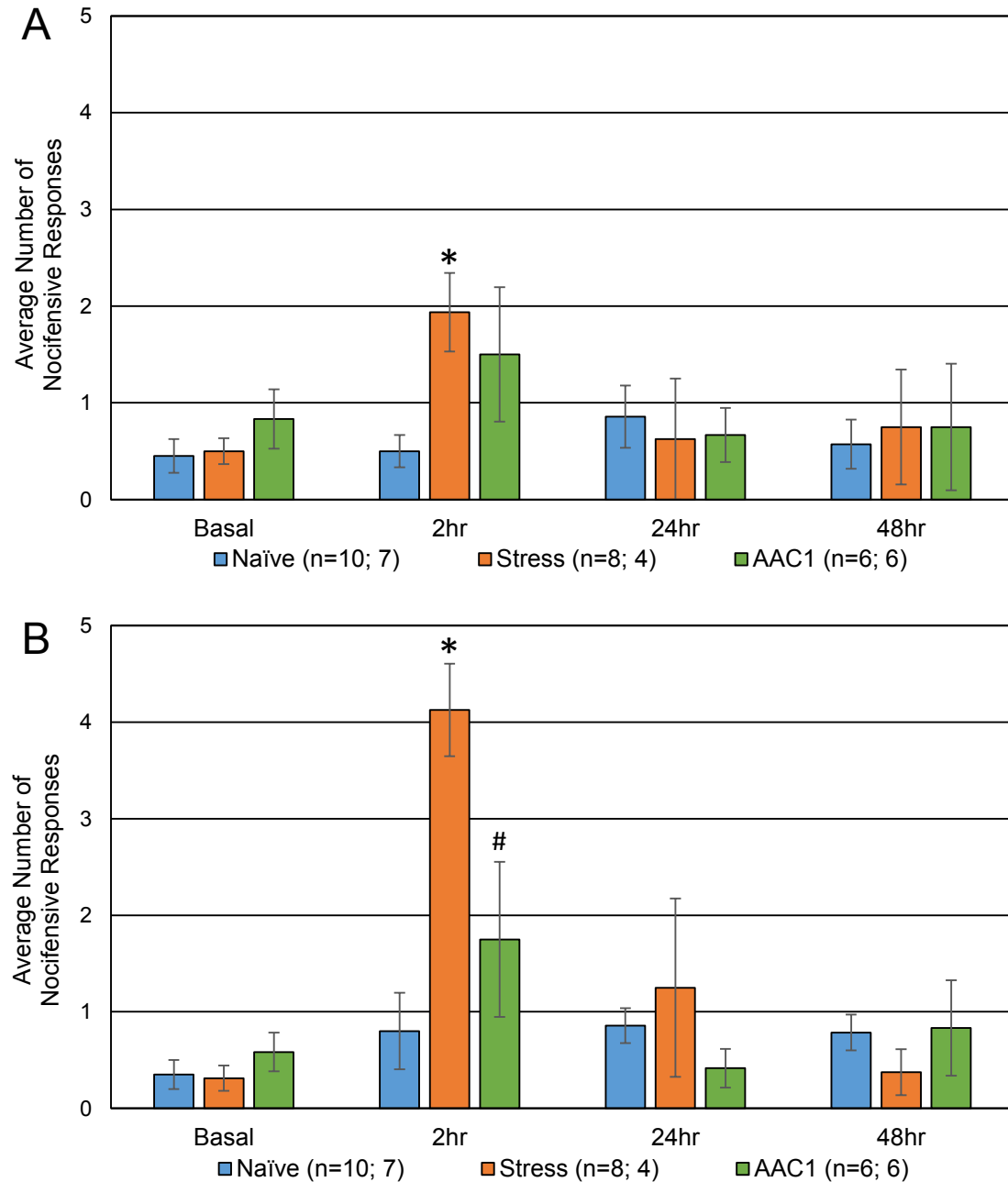


Figure 11. Orofacial sensitization resolves one day post exposure to migraine trigger in male offspring exposed to early life stress. The number of animals at the 24hr and 48hr time points is reported following the semicolon since fewer animals were available when compared to naïve and 2hr time points. 11A shows V1 level of sensitization and 11B shows V3 sensitization. The error bars are \pm SEM. A significant increase from the naïve animals is indicated by an asterisk while a significant decrease from stressed animals is indicated by a pound sign.

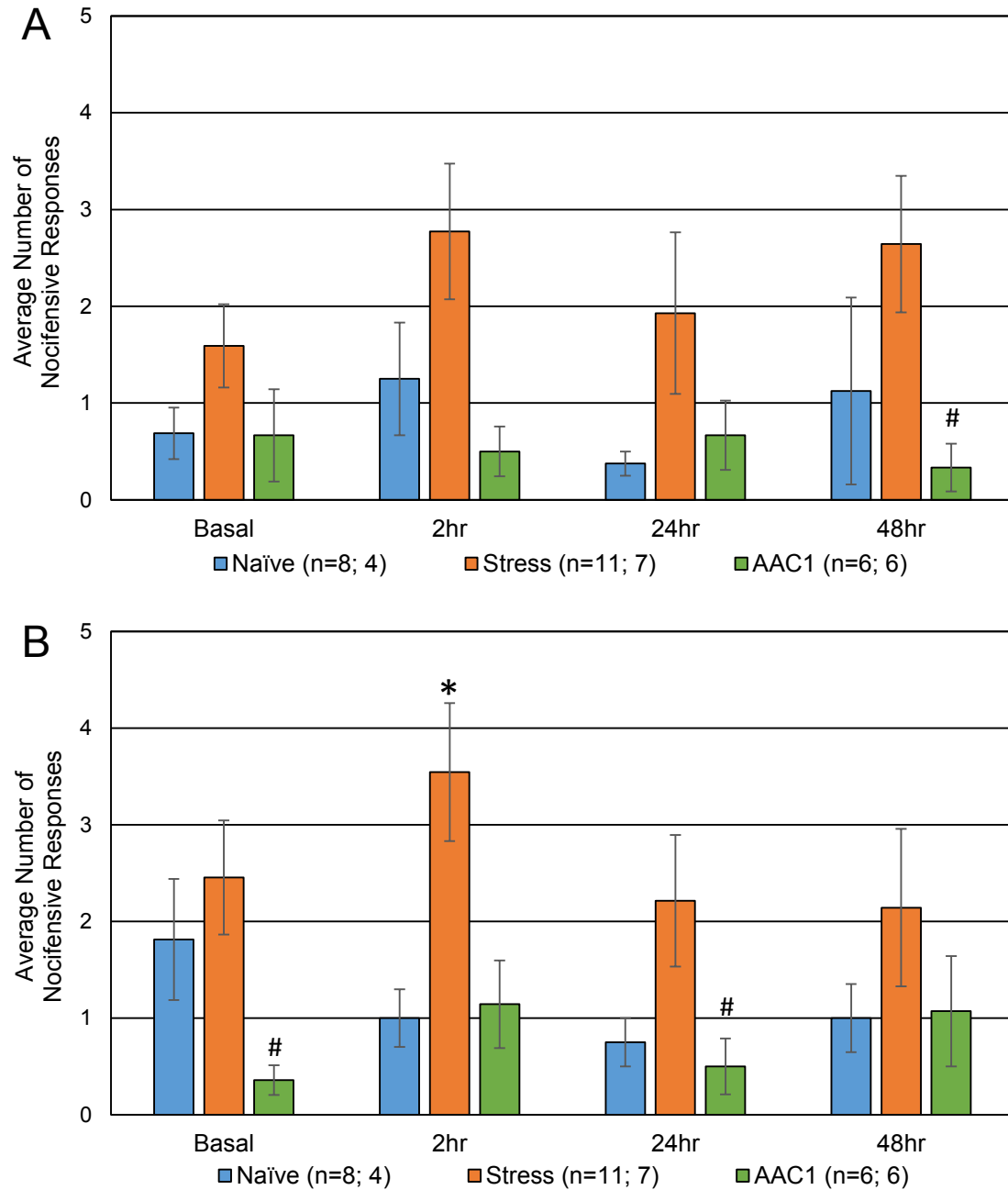


Figure 12. Orofacial sensitization does not resolve two days post exposure to migraine trigger in female offspring exposed to early life stress. The number of animals at the 24hr and 48hr time points is reported following the semicolon since fewer animals were available when compared to naïve and 2hr time points. 12A shows V1 level of sensitization and 12B shows V3 sensitization. The error bars are \pm SEM. A significant increase from the naïve animals is indicated by an asterisk while a significant decrease from the stressed animals is indicated by a pound sign.

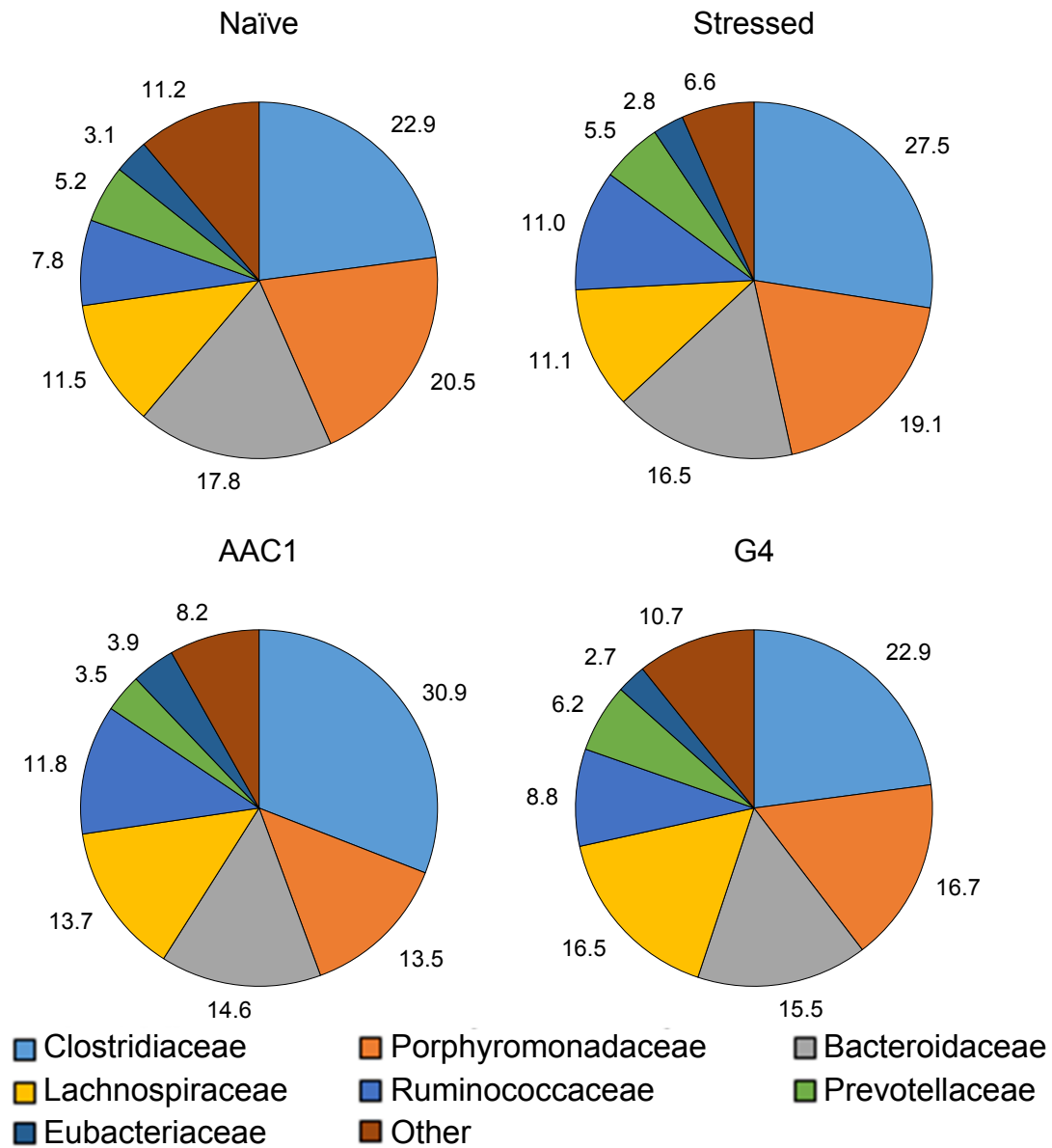


Figure 13. Family abundance from male offspring fecal samples. Displayed numbers are the percent abundance of a family within the sample.

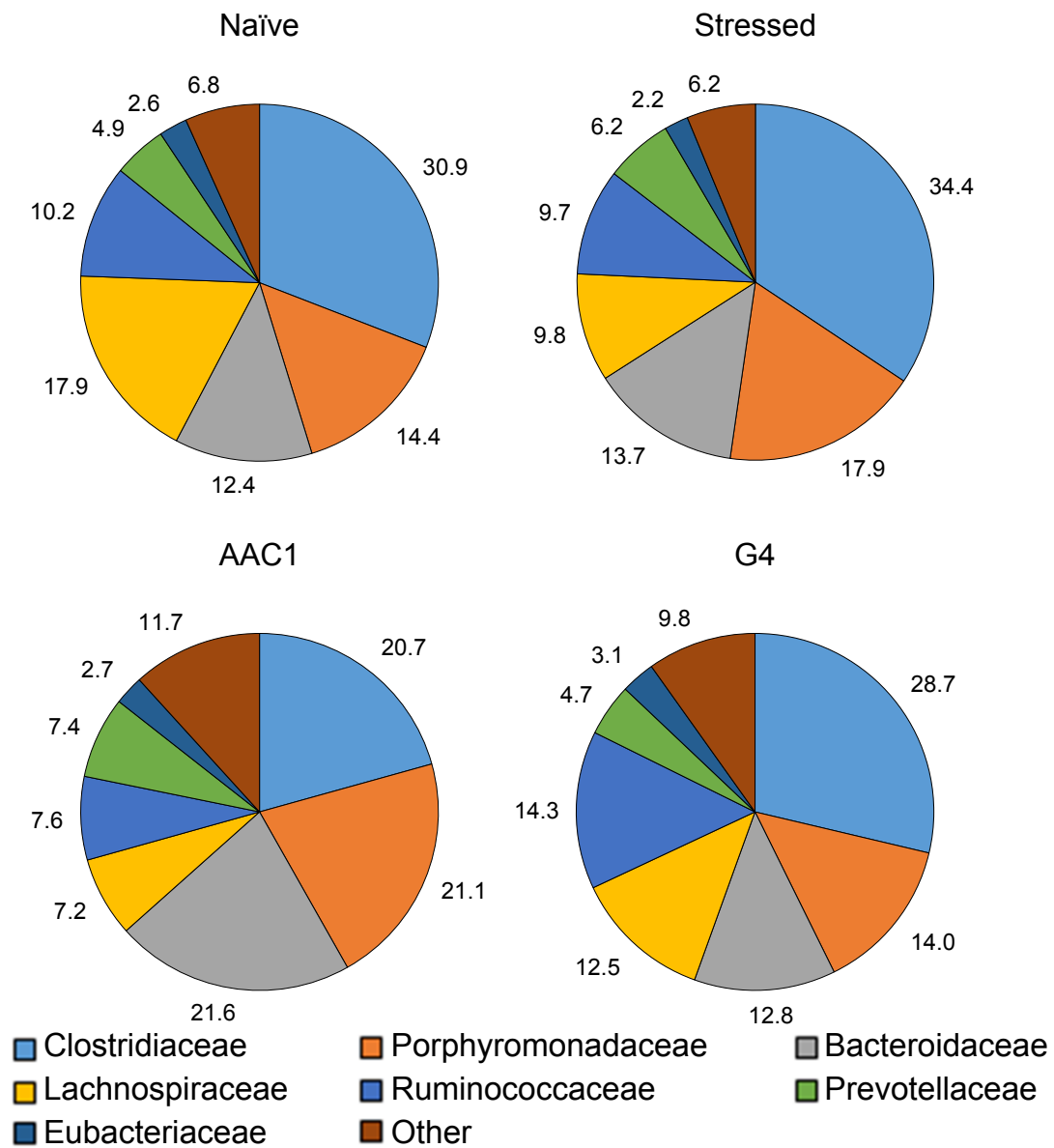


Figure 14. Family abundance from female offspring fecal samples. Displayed numbers are the percent abundance of a family within the sample.

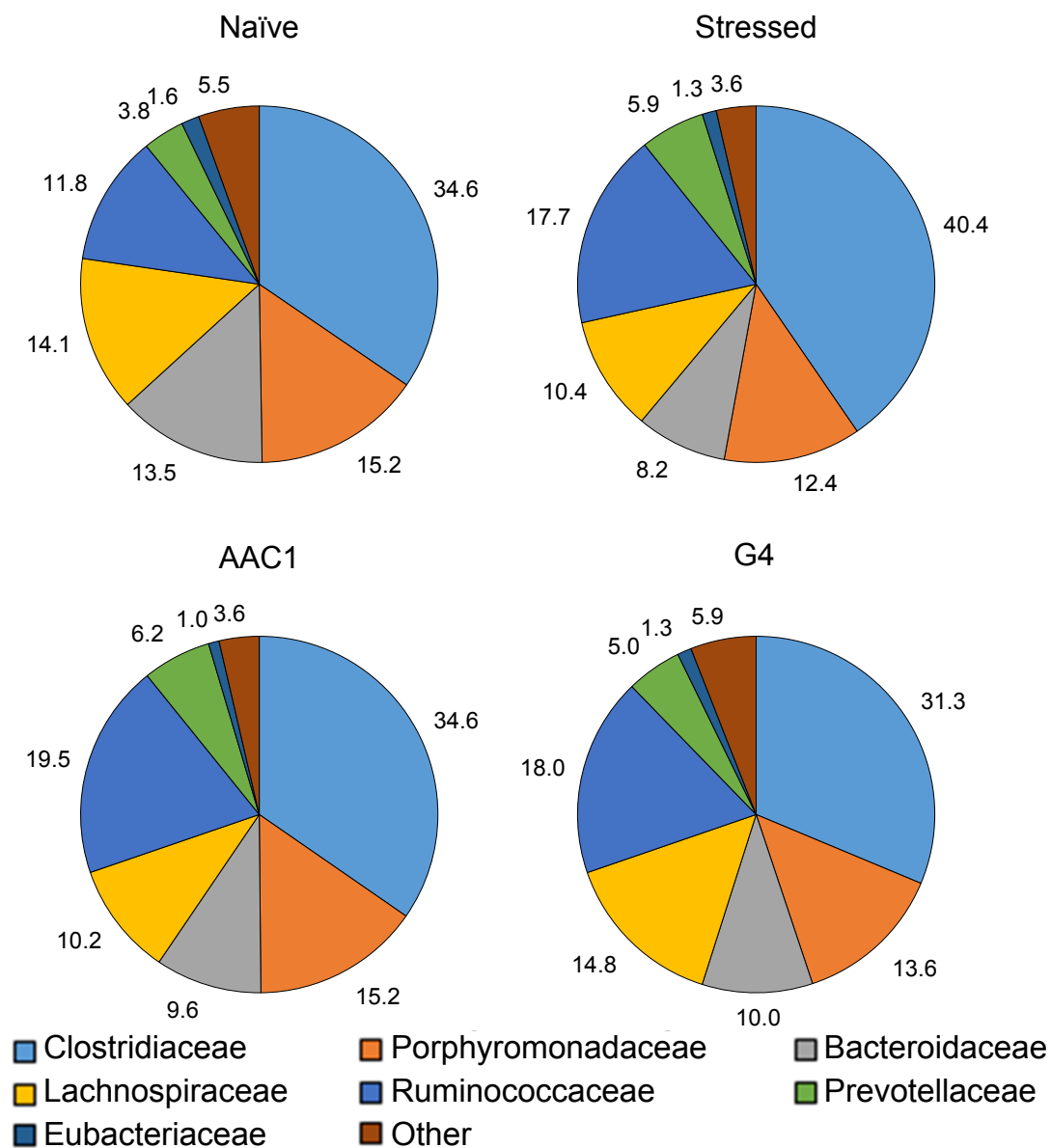


Figure 15. Family abundance from male offspring cecum samples. Displayed numbers are the percent abundance of a family within the sample.

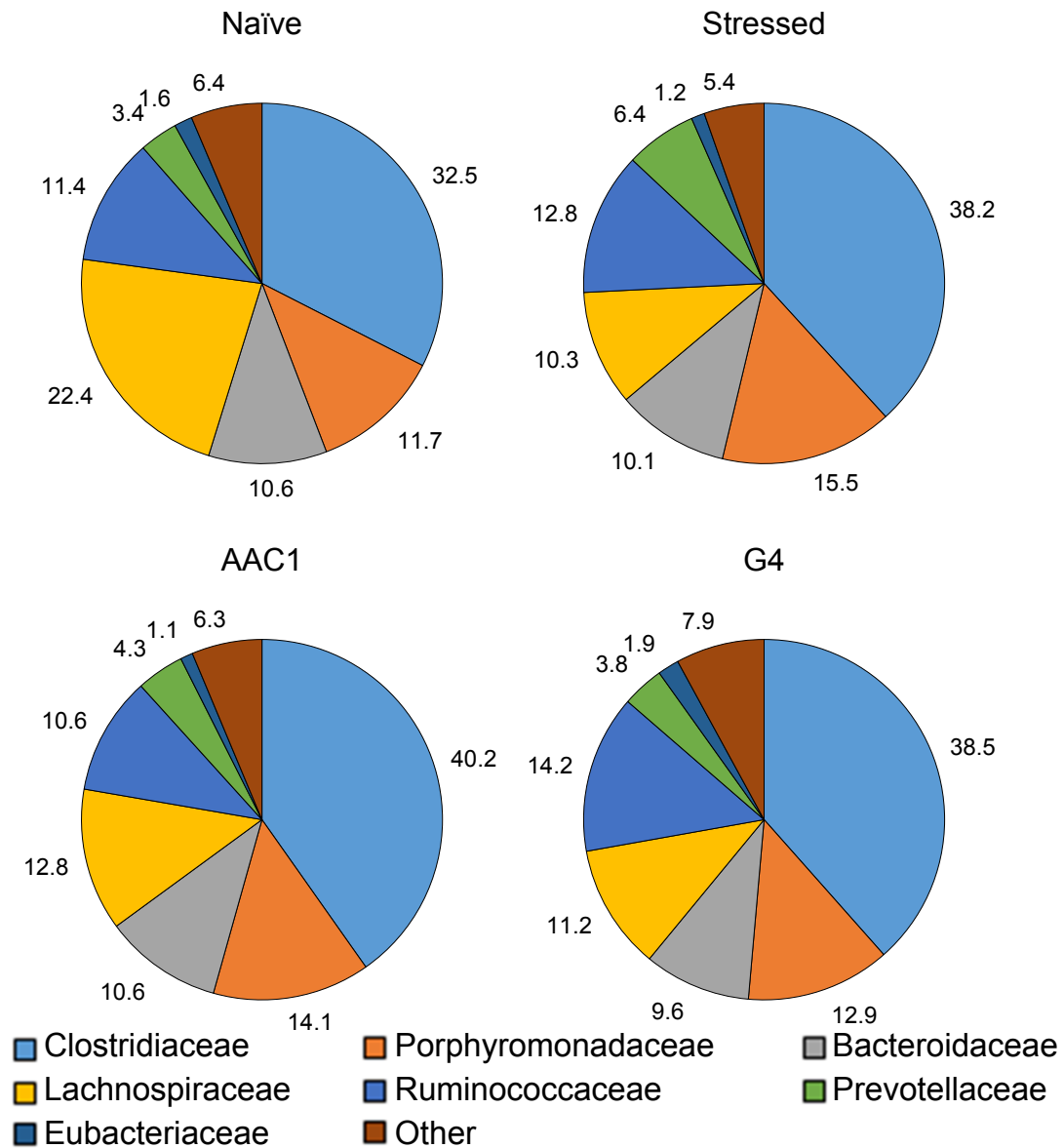


Figure 16. Family abundance from female offspring cecum samples. Displayed numbers are the percent abundance of a family within the sample.

Table 2. Relative abundance of the most prevalent genera in male offspring fecal samples. Blue highlighted data indicate a 3% or more increase of total abundance of a genus or a greater than a 2 fold increase when compared to the naïve. Yellow highlighted data indicate a 3% or more decrease of total abundance of a genus or a greater than 2 fold decrease when compared to naïve.

Genus	Percent Abundance of Genera			
	Naïve	Stressed	AAC1	G4
<i>Clostridium</i>	22.5	27.0	30.5	22.7
<i>Bacteroides</i>	17.8	16.5	14.6	15.5
<i>Barnesiella</i>	13.1	12.9	7.9	10.5
<i>Oscillospira</i>	3.9	6.6	6.8	4.2
<i>Prevotella</i>	5.2	5.5	3.5	6.2
<i>Tannerella</i>	5.3	4.4	4.3	4.3
<i>Lachnospirillum</i>	6.2	2.4	2.2	2.3
<i>Robinsoniella</i>	1.1	2.8	3.7	4.6
<i>Eubacterium</i>	3.1	2.8	3.9	2.7
<i>Ruminococcus</i>	1.7	3.0	2.6	3.7
<i>Blautia</i>	1.5	2.0	3.9	4.5
<i>Parasutterella</i>	2.1	1.2	1.3	3.0
<i>Coproccoccus</i>	0.5	0.8	1.0	2.8
<i>Kopriimonas</i>	1.1	0.4	1.8	1.0
<i>Pseudoflavonifractor</i>	0.9	0.5	0.6	0.6

Table 3. Relative abundance of the most prevalent genera in female offspring fecal samples. Blue highlighted data indicate a 3% or more increase of total abundance of a genus or a greater than a 2 fold increase when compared to the naïve. Yellow highlighted data indicate a 3% or more decrease of total abundance of a genus or a greater than 2 fold decrease when compared to naïve.

Genus	Percent Abundance of Genera			
	Naïve	Stressed	AAC1	G4
<i>Clostridium</i>	30.6	33.8	20.5	28.2
<i>Bacteroides</i>	12.4	13.7	21.6	12.8
<i>Barnesiella</i>	9.9	11.1	14.1	8.4
<i>Oscillospira</i>	6.9	6.8	3.2	6.4
<i>Prevotella</i>	4.9	6.2	7.4	4.7
<i>Tannerella</i>	3.2	5.2	5.0	3.7
<i>Lachnospirillum</i>	7.1	2.4	1.9	3.2
<i>Robinsoniella</i>	6.5	1.1	2.0	5.4
<i>Eubacterium</i>	2.5	2.2	2.6	3.0
<i>Ruminococcus</i>	0.9	1.1	3.2	5.5
<i>Blautia</i>	1.7	2.4	0.9	0.9
<i>Parasutterella</i>	1.0	0.7	2.1	1.3
<i>Coproccoccus</i>	0.8	2.2	0.6	0.5
<i>Kopriimonas</i>	0.5	0.9	1.3	1.0
<i>Pseudoflavonifractor</i>	1.6	0.6	1.2	1.3

Table 4. Relative abundance of the most prevalent genera in male offspring cecum samples. Blue highlighted data indicate a 3% or more increase of total abundance of a genus or a greater than a 2 fold increase when compared to the naïve. Yellow highlighted data indicate a 3% or more decrease of total abundance of a genus or a greater than 2 fold decrease when compared to naïve.

Genus	Percent Abundance of Genera			
	Naïve	Stressed	AAC1	G4
<i>Clostridium</i>	33.9	39.7	33.4	30.8
<i>Oscillospira</i>	8.8	14.7	14.9	15.4
<i>Bacteroides</i>	13.5	8.2	9.6	10.0
<i>Barnesiella</i>	10.0	7.6	9.2	8.4
<i>Prevotella</i>	3.8	5.9	6.2	5.0
<i>Lachnoclostridium</i>	7.4	3.0	2.4	2.6
<i>Tannerella</i>	4.0	3.7	5.0	3.8
<i>Robinsoniella</i>	2.4	1.1	1.8	2.3
<i>Blautia</i>	2.3	2.8	2.6	4.1
<i>Eubacterium</i>	1.6	1.2	0.9	1.2
<i>Coproccoccus</i>	0.3	0.6	1.5	1.9
<i>Anaerotruncus</i>	0.7	1.1	1.1	0.9
<i>Pseudoflavonifractor</i>	0.9	0.5	0.7	0.9
<i>Ruminococcus</i>	0.8	0.7	0.7	1.1
<i>Anaerostipes</i>	0.3	0.8	0.6	1.7

Table 5. Relative abundance of the most prevalent genera in female offspring cecum samples. Blue highlighted data indicate a 3% or more increase of total abundance of a genus or a greater than a 2 fold increase when compared to the naïve. Yellow highlighted data indicate a 3% or more decrease of total abundance of a genus or a greater than 2 fold decrease when compared to naïve.

Genus	Percent Abundance of Genera			
	Naïve	Stressed	AAC1	G4
<i>Clostridium</i>	32.0	37.4	39.7	37.7
<i>Oscillospira</i>	8.2	10.2	8.1	11.0
<i>Bacteroides</i>	10.6	10.1	10.6	9.6
<i>Barnesiella</i>	8.0	9.6	9.2	6.9
<i>Prevotella</i>	3.4	6.4	4.3	3.8
<i>Lachnoclostridium</i>	8.5	3.0	3.0	3.4
<i>Tannerella</i>	2.6	4.5	3.7	4.6
<i>Robinsoniella</i>	9.3	1.6	4.3	2.9
<i>Blautia</i>	2.0	1.9	2.2	1.5
<i>Eubacterium</i>	1.6	1.2	1.1	1.9
<i>Coproccoccus</i>	0.8	1.8	0.4	1.0
<i>Anaerotruncus</i>	0.6	0.9	0.7	1.1
<i>Pseudoflavonifractor</i>	1.4	0.9	0.8	1.0
<i>Ruminococcus</i>	1.0	0.7	0.7	1.0
<i>Anaerostipes</i>	0.6	0.5	1.6	0.8

DISCUSSION

Early Life Stress Promotes Sustained Trigeminal Sensitization

Sensitization or a lowering of the activation threshold of trigeminal neurons is implicated in the underlying pathology of migraine and other orofacial pain conditions such as TMD that are more prevalent in females than males (Chaves et al., 2016; Chichorro et al., 2017; de Tommaso and Sciricchio, 2016). In my thesis project, I tested the hypothesis that exposure to secondary traumatic stress during early development would lead to enhanced nocifensive head withdrawal response to mechanical stimulation of the V1 and V3 trigeminal regions. In addition, I investigated whether females would exhibit an increased basal level of sensitivity. The foundation for my proposed experiments was based on results from a prior study in our laboratory that early life stress resulted in a sustained elevation in the levels of several key pro-inflammatory and pro-nociceptive signaling proteins in the trigeminal nerve and spinal trigeminal nucleus (Hawkins et al., 2018). Importantly, increased levels of mitogen activated protein kinases in these regions are implicated in the initiation and maintenance of peripheral and central sensitization (Ji et al., 2009), which are physiological changes in the nervous system involving enhanced communication between neurons and glial cells (Dodds et al., 2016; Milligan and Watkins, 2009; Wieseler-Frank et al., 2004). Migraine and TMD pathology are associated with peripheral and central sensitization that can progress to a primed state of both primary and secondary nociceptive neurons via prolonged cellular changes in the expression of ion channels, receptors, and signaling pathways (Hucho and Levine, 2007). Results from my study provide evidence that while early life stress does not significantly

change the basal nocifensive response in the V1 (temporalis) region, there was a significant increase in sensitivity in females in the V3 (masseter) region. Although not a focus of my study, it would be of interest to determine if early life stress may be a risk factor for the development of TMD especially in females given the increased mechanical sensitivity in the masseter muscle, which is a common site of pain with TMD (Lobbezoo et al., 2004). Previous unpublished findings from our laboratory have shown that neck muscle inflammation, which mediates trigeminal sensitization, is a risk factor for the development of a persistent hyperexcitable state following prolonged jaw opening. Future studies could be directed to determine if early life stress will increase the risk of developing chronic TMD pathology when combined with other risk factors such as neck muscle inflammation, sleep deprivation, gender, and prolonged jaw opening (Fillingim et al., 2013; Greenspan et al., 2013; Slade et al., 2016).

To determine whether early life stress may promote a sensitized state of trigeminal nociceptors and thus function as a risk factor for migraine, I utilized a model of an episodic migraine attack that involves trigeminal nociceptor activation in a sensitized animal following exposure to a pungent odor reported to be a migraine trigger in humans (Hawkins et al., 2017). Both male and female offspring exposed to early life stress exhibited an increase in the average number of nocifensive responses to mechanical stimulation of the V1 and V3 regions two hours post exposure to the migraine trigger. Interestingly, while the level of sensitization returned to basal levels in male animals 24 hours after the odorant trigger, female animals exhibited increased nociceptive responses in V1 and V3 regions at 24 and 48 hours, which is indicative of a sensitized trigeminal system. My results support the notion that early life stress may function as a migraine risk

factor by lowering the activation threshold of trigeminal neurons indicative of a primed nociceptive state that underlies chronic pain conditions (Hucho and Levine, 2007). The fact that I observed increased sensitivity in the temporalis and masseter muscles may be clinically relevant since migraineurs often report increased pain (hyperalgesia) as well as allodynia, a painful response to a normally non-painful stimulus, in their head and face (Cady et al., 2009; Misra et al., 2013). Furthermore, my findings are in agreement with data from human studies that early life stress is associated with an increased risk of migraine and other orofacial pain conditions and female gender should be considered a risk factor (Ohrbach et al., 2011; Slade et al., 2013; Tietjen and Peterlin, 2011; Tietjen et al., 2012; Tietjen, 2016).

A major goal of my study was to determine whether inclusion of an enriched chicken bone broth as a dietary supplement could inhibit sensitization of trigeminal nociceptors and prevent their activation by a known migraine trigger. I found that daily inclusion of an enriched chicken bone broth was sufficient to mediate a decrease in the basal level of sensitivity in female, but not male animals, and suppressed trigeminal nociceptor activation in response to the migraine trigger in both males and females. However, animals that received daily supplementation with a homemade chicken bone broth, described in detail in the publication by Hawkins et al., (Hawkins and Durham, 2018), did not inhibit trigeminal nociceptor sensitivity. My finding agrees with results from a prior study in our laboratory that dietary inclusion of an enriched chicken bone broth was sufficient to inhibit trigeminal activation in a model of TMD caused by prolonged jaw opening (Hawkins and Durham, 2018). Furthermore, the results with respect to the beneficial properties of bone broth to inhibit trigeminal nociceptor

activation is consistent with the reported inhibitory effects mediated by dietary inclusion of cocoa (Cady and Durham, 2010; Cady et al., 2013) and grape seed extract (Cady et al., 2010). Given the opioid epidemic and our overdependence on all types of drugs to relieve pain, the identification of novel, more natural therapeutic strategies are greatly needed to inhibit pain pathways and minimize unwanted negative side effects. Taken together, findings from our laboratory have demonstrated in clinically relevant rat models that dietary inclusion of natural products provides a novel non-pharmacological method for modulating the trigeminal system and thus reducing the risk of developing migraine or TMD later in life.

An important question to address is the possible mechanism by which the enriched chicken bone broth used in my study regulates the excitability state of trigeminal neurons. Based on previous work from our laboratory, the chicken bone broth obtained from International Dehydrated Foods, Inc., was enriched in compounds that exhibit antioxidant activity/reducing potential and inhibit synthesis of pro-inflammatory prostaglandins via selective inhibition of the cyclooxygenase enzyme II (COX II) but not COX I (Hawkins and Durham, 2018). In addition, the bone broth was found to inhibit the upregulation of protein kinase A, a signaling protein known to promote peripheral and central sensitization in trigeminal nociceptive neurons. Thus, it is possible that each of these properties of the bone broth may be partly responsible for the observed inhibitory effect in the episodic migraine model. However, it is also plausible that the anti-nociceptive effect of the bone broth may be due, at least in part, to its ability to influence the gut microbiota to favor bacteria that produce anti-inflammatory molecules. There is a growing body of literature to support the notion that a healthy nervous system is linked to

a healthy gut (Aaron et al., 2000; Rea et al., 2016; Rea et al., 2017; Yang and Chiu, 2017). While much of the focus of our diet is on foods that provide nutritional value to us directly, it is equally important to our overall health to maintain a diverse gut flora. Many of the insoluble products in our diet such as collagen and other types of fiber provide nutrition to bacteria in our intestines and colon. Interestingly, most chronic pain conditions are comorbid with irritable bowel syndrome and migraine pathology (Camara-Lemarroy et al., 2016), and migraine was recently reported in a genome-wide association study to be more similar to irritable bowel syndrome than other neurological diseases (Wang et al., 2017). This information is consistent with the fact that both migraine, TMD, and irritable bowel syndrome are considered inflammatory diseases (Camara-Lemarroy et al., 2016). In addition to our own innate ability to modulate neuroimmune interactions within the nervous system, bacteria in our gut can synthesize and release metabolites (pharmabiotics) systemically that regulate inflammatory events in the gut, which are associated with modulating the excitability state of peripheral and central neurons (Patterson et al., 2014). For example, native bacteria found in our gut are able to produce metabolites such as the inhibitory neurotransmitter GABA that helps to maintain a normal excitability state of nociceptive neurons by inhibiting ascending painful signaling mediated by the excitatory neurotransmitter glutamate. Another metabolite produced by gut bacteria is the neurotransmitter serotonin or 5-HT that functions to modulate pain signaling via activation of the descending pain pathway involving the paraventricular nucleus, periaqueductal grey region, and second order neurons in the spinal cord. Additionally, gut bacteria can synthesize short chain fatty acids such as propionate and butyrate, which function to maintain homeostasis within the intestine but also have been

shown to exert anti-inflammatory effects in the nervous system. Importantly, receptors for short chain fatty acids have been reported to be expressed in trigeminal ganglion neurons, which could provide a plausible direct mechanism for regulating the excitability state of primary nociceptors. Activation of these receptors has been shown to couple to the G_i/G_o pathway to inhibit the cAMP/Protein Kinase A pathway, which is implicated in neuronal sensitization of trigeminal neurons (Koop et al., 2017; Seybold, 2009).

Effect of Early Life Stress and Enriched Chicken Bone Broth on Gut Microbiota

A somewhat surprising finding from my study was the lack of any major shifts in the gut microbiota such as the Bacteroides to Firmicutes (B/F) ratio, which would have been indicative of dysbiosis and an altered gut-nervous system axis. Similarly, major differences in the bacterial population were not seen when comparing fecal vs cecum samples. However, I did observe differences between female and male bacterial profiles at the genus level in fecal samples from animals provided enriched chicken bone broth and exposed to early life stress, indicating that the enriched chicken bone broth may promote different bacteria in the colon based on the sex of the organism. Additional small changes in the abundance levels were seen at the genus level, but the impact of these shifts with respect to trigeminal sensitization is difficult to interpret given the complexity of the interactions between various bacterial species. A plausible explanation for the minor changes in microbiota is that the early life stress model, although sufficient to lower trigeminal nociceptor sensitivity, was not severe enough to cause significant dysbiosis that would be detectable in a pooled sample population. Based on the comorbid association of chronic migraine and irritable bowel syndrome, is likely that in a more

chronic pain state involving persistent hyperactivity of trigeminal neurons, I would have observed more pronounced shifts in the gut microbiota. Alternatively, future studies could utilize Illumina technology to look at individual profiles, which may be required to provide a more accurate analysis and identify key bacterial changes to help explain the increased sensitivity seen with early life stress and inhibition with chicken bone broth.

Although I cannot determine if the changes seen in my study were statistically significantly different due to pooling the samples, my findings were not similar to changes reported in other rodent studies. In a model of prenatal stress during the last week of gestation, male Sprague-Dawley rats' fecal microbiota profiles were assessed at 4 months of age (Golubeva et al., 2015). In that study, they found that prenatal stress increased bacteria in the *Oscillibacter*, *Anaerotruncus*, and *Peptococcus* genera. In my study, early life stress shifted the microbiota towards higher levels of *Oscillibacter* and *Anaerotruncus* with a small decrease in *Peptococcus* (data not shown). Another study looking at acute tail shock stress in young adult male Fischer 344 rats (>10 weeks of age) reported a decrease in *Prevotella* in response to acute stress in fecal and cecum samples (Maslanik et al., 2012), while I observed an increase in *Prevotella* in response to early life stress (Tables 2 and 4). The findings from a study of the effects of a social stressor on the male cecum of mice were different from my findings (Bailey et al., 2011). In that study, young adult mice (>10 weeks of age) that were exposed to an aggressive mouse that acted as a social stressor exhibited a decrease in *Pseudobutyrovibrio*, *Dorea*, and *Coprococcus*. It is important to note that besides *Prevotella*, the relative abundance of these genera were often below 1% and so may not exert much of a physiological change in the gut or nervous system. A study looking at the effects of limited nesting stress in

young Wistar rat offspring's (postnatal day 21) fecal microbiota found 44 genera to be significantly different from the naïve (Moussaoui et al., 2017). Interestingly, there were no statistically significant differences between young male and young female offsprings' microbiota. While some results from that study were similar to my results such as the finding that limited nesting stress increased *Clostridium* and *Coprococcus*, all other results from my study were opposite to their results, had small differences between stressed and naïves, or were not in the top 15 most abundant genera (Table 2). In a study investigating the effects of long term dietary inclusion of various protein diets in 16 week old Sprague-Dawley males, animals fed a chicken protein diet had the highest B/F ratio and *Lactobacillus* abundance between all of the groups (Zhu et al., 2017). My results show a decrease in B/F ratio when comparing AAC1 to early life stress with water, and little difference between G4 and early life stress with water. Additionally, *Lactobacillus* was unaffected by inclusion of AAC1 or G4 in male fecal samples. The lack of agreement between results from my study and other published findings could be due to multiple factors including differences in the age of the animals, species of rodent, type and severity of stress, and different diets.

Multiple studies have shown that stressed male mice or Sprague-Dawley have a lower amount of *Lactobacillus* in their fecal samples (Bailey et al., 2011; Golubeva et al., 2015; Jasarevic et al., 2017; Marin et al., 2017). In my study, the naïve male fecal microbiota was comprised of 0.68% *Lactobacillus*, while the animal receiving water and exposed to early life stress had 1.16% *Lactobacillus* (data not shown). Because the samples were pooled in my study, it is possible that a single animal from the naïve or the stressed group could be shifting the average down or up, respectively. Additionally, the

male animals do not exhibit statistical differences basally, and only animals receiving early life stress and water became sensitized when exposed to a known migraine trigger at the two hour time point. This finding provides evidence to support the notion that my model is not as severe as the stress models from other studies that investigated the effects of stress on the microbiota. However, the female animals in my study do exhibit this shift in *Lactobacillus* that is often reported in male rodents. In both the female fecal and cecum samples, *Lactobacillus* was decreased in the early life stress with water animals and increased in animals exposed to early life stress and receiving either AAC1 or G4 bone broths.

Summary and Future Directions

In summary, results from my studies have provided evidence that early life exposure to secondary traumatic stress is sufficient to promote a sensitized state of trigeminal nociceptive neurons that lowers their activation threshold to a known migraine trigger. The level of sensitivity and activation was greater in female offspring versus male offspring. Importantly, dietary supplementation with an enriched chicken bone broth was shown to inhibit trigeminal sensitization and block activation of trigeminal neurons in a model of episodic migraine. The model of early life stress utilized in my thesis research involved exposure of the breeding parents (mother and father), pregnant mothers, and their offspring to the primary stressor animal. Because of this experimental design, I do not know which of the exposures was most responsible for the phenotypic changes in nociceptive sensitivity and cellular changes in the trigeminal ganglion and spinal trigeminal nucleus. It would be interest to investigate the impact of secondary exposure to

only the father, only the mother at either time point, or only the offspring in future studies. In this manner, the mechanism by which male and female offspring are differentially imprinted for a sensitized trigeminal system could be determined. Since the phenotypic changes are likely to involve epigenetic modifications, future studies could be directed to determine the role of DNA methylation, histone modifications, and microRNA expression under each of the conditions. Another area of future study could involve the use of immunohistochemistry to determine the cellular changes in response to activation of trigeminal neurons with the bay leaf extract in trigeminal ganglion and spinal trigeminal nucleus. Finally, it would be of interest to test the hypothesis that the production of short chain fatty acids by gut bacteria may be directly involved in suppressing trigeminal sensitization and activation via activation of their receptors on trigeminal nociceptive neurons.

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