THE EFFECT OF CANNABINOIDS ON VAGAL NODOSE ACTIVATION AFTER TREATMENT WITH CHOLECYSTOKININ OR LIPOPOLYSACCHARIDE

by

JULIANE ROSE JOHNSTON

(Under the Direction of Gaylen Edwards)

ABSTRACT

The vagus nerve is the tenth cranial nerve, and it is involved in function of the respiratory, cardiovascular, and gastrointestinal systems. Vagal afferent neurons are capable of sensing many factors about the environment through a number of different receptors. These receptors include cholecystokinin (CCK) receptors, which are involved in satiety, cannabinoid receptors, which are involved in satiety and inflammation, and TLR-4 receptors that bind to lipopolysaccharide (LPS) and induce inflammatory processes. All three of these receptors are located on vagal afferent neurons. The purpose of this project is to discover more about their effects on neuronal activation and the signal transduction pathways that are involved.

This dissertation details 1) the effect of a cannabinoid agonist, CP 55,940, on neuronal activation by CCK in cultured vagal afferent neurons using immunohistochemistry, 2) the effect of CP 55,940 on neuronal activation by LPS in cultured vagal afferent neurons using immunohistochemistry, and 3) the effect of CP 55,940 on neuronal activation by LPS on pNF-

κB, NF-κB, and SOCS-3 expression in whole nodose ganglia when treated *in vitro* using Western blotting.

The results of these studies have shown that pre-treatment with a cannabinoid receptor agonist, CP 55,940, causes a decrease in activation by CCK in vagal afferent neurons in culture. In addition, it has been shown that pre-treatment with CP 55,940 causes a decrease in vagal afferent neuron activation by LPS in culture. Lastly, the data have shown that LPS causes a decrease in cytoplasmic pNF-κB and NF-κB and an increase in SOCS-3 expression in nodose ganglia *in vitro* and that CP 55,940 causes a decrease in SOCS-3 expression after stimulation with LPS but does not cause a change in pNF-κB or NF-κB expression. Collectively, these studies have shown the effects of cannabinoids on vagal afferent neuronal activation after treatment with two different stimuli and have aided in elucidating the signal transduction pathways involved. This indicates that cannabinoids may be used as therapeutic agents to increase food-seeking behavior or to decrease vagal afferent activation during an inflammatory state.

INDEX WORDS: Cannabinoids, Cholecystokinin, Lipopolysaccharide, Vagus, NF-κB, Inflammation, Cytokines, Satiety

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DEDICATION

Dedicated to
my mother, Janis Carol Rose,
for her support, love, and encouragement throughout the whole grueling process

and my husband, Alexander James Johnston,
for loving and supporting me and for the sacrifices that he has made to make this possible

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Abbreviations

2-arachidonoyl-glycerol (2-AG), adrenocorticoptropic hormone (ACTH), agouti-related peptide (AGRP), anandamide (AEA), arcuate nucleus (ARC), area postrema (AP), calcitonin gene-related peptide (CGRP), cholecystokinin (CCK), cocaine-and-amphetamine regulated transcript (CART), corticotrophin-releasing hormone (CRH), dorsal medullary ventricle (DMV), dorsomedial hypothalamus (DMH), dorsomedial nucleus (DMN), dipeptidyl peptidase-4 (DPP4), dorsal root ganglion (DRG), fatty acid amide hydrolase (FAAH), galanin-like peptide (GALT), gamma-aminobutyric acid (GABA), glucagon-like peptide (GLP), insulin receptor substrate (IRS), interleukin-1-beta (IL-1β), irritable bowel disease (IBD), lateral hypothalamic area (LHA), lateral parabrachial nucleus (LPBN), lipopolysaccharide (LPS), melanin-concentrating hormone (MCH), melanocyte-stimulating hormone (MSH), N-methyl-D-aspartate (NMDA), neuropeptide Y (NPY), nuclear factor – κB (NF-κB), nucleus of the solitary tract (NTS), parabrachial nucleus (PBN), paraventricular nucleus (PVN), phospholipase C (PLC), pro-opiomelanocortin (POMC), protein kinase C (PKC), serotonin (5-HT), suppressor of cytokine signaling-3 (SOCS-3), suprachiasmatic nucleus (SCN), thyrotropin-releasing hormone (TRH), tumor necrosis factor-alpha (TNF-α), vanilloid receptor-1 (VR_1), ventrolateral medulla (VLM), ventromedial nucleus of the hypothalamus (VMH)

Section I: Introduction

The vagus nerve is known as the "wanderer" and innervates many organs. It has both sensory and motor functions within the body, and it is involved in the function of the cardiovascular, respiratory, and gastrointestinal systems[1]. Vagal afferent fibers carry sensory information from the upper gastrointestinal tract to the brainstem. Many different chemical stimuli are involved in integrating information from the gastrointestinal tract to the central nervous system, including cholecystokinin (CCK) and cannabinoids.

CCK is an anorexigenic hormone that is released and binds to CCK receptors in response to food intake. Endocannabinoids are endogenous ligands for the CB receptors[2]. Endocannabinoids are reportedly orexigenic hormones that encourage feeding during a state of fasting[3]. They have also been shown to have anti-inflammatory effects on the brain and in other systems[4]. The intention for this project is to further describe the effects of cannabinoids on vagal afferent neurons when they are treated with CCK or lipopolysaccharide (LPS), a bacterial endotoxin. Moreover, this project tests the hypothesis that LPS will activate neurons in the nodose ganglia. LPS increases production of cytokines such as TNF-α and IL-1β through increased expression of NF-κB[5], so another aim of this project is to evaluate the role of a cannabinoid agonist in decreasing activation of nodose ganglion neurons by LPS by decreasing the expression of NF-κB. The overall goal of this project is to provide novel insights into the potential that behavioral changes involving vagal afferent fibers are altered by cannabinoids. We hypothesize that vagal afferent fibers are activated both by CCK and endotoxin and that

this activation can be inhibited by cannabinoids *in vitro*. These experiments provide more knowledge about the mechanism through which cannabinoids work in vagal afferent fibers. This knowledge may be used therapeutically in diseases such as irritable bowel syndrome and Crohn's disease.

The goal of this project will be met by achieving the following **specific aims**:

AIM 1 To examine the effect of a cannabinoid agonist on activation of vagal afferent neurons from the nodose ganglia by CCK *in vitro*

AIM 2 To examine the effect of a cannabinoid agonist on inflammation caused by LPS in vagal afferent neurons *in vitro*

AIM 3 To examine the effect of a cannabinoid agonist on NF-κB and SOCS-3 expression caused by LPS in whole nodose ganglia

Section II: Basic Vagal and Gastrointestinal Neuroanatomy

The vagus nerve is the tenth cranial nerve in the nervous system, and it innervates areas of the body from the upper esophagus to the transverse colon[6]. In Latin, the word vagus means "wanderer," and it refers to its extensive innervation of many organs of the body, including the heart, thymus, lungs, liver, and the gastrointestinal tract. The nerve also contains dendritic cells that aid in the function of the immune system[1]. The vagus nerve is made up of both myelinated neurons with large diameters and non-myelinated neurons. Approximately eighty percent of the fibers are non-myelinated. These axons are packed together tightly[7].

Anatomical Pathway of the Vagus Nerve

The axons of the general somatic efferent fibers of the vagus nerve originate in the center of the nucleus ambiguus, and they exit the brain at the lateral edge of the medulla rostral to the internal branch of the accessory nerve. These fibers then travel with the general vagal efferents that originate in the parasympathetic nucleus of the vagus[8]. The preganglionic neurons in the parasympathetic nucleus of the vagus are organized spatially. Those that innervate the abdominal organs are located in the rostral region. Those in the middle supply the abdominal and thoracic regions, while those in the caudal region innervate cervical viscera, generally[9]. The axons from the vagus and accessory nerves join to form the motor portion of cranial nerve X[8]. The vagus nerve leaves the skull through the jugular foramen[8]. The proximal ganglion (or jugular) lies within the foramen[9].

Once the vagus passes through the jugular foramen, the auricular branch runs through the petrous temporal bone laterally to join with the facial nerve. This portion of the vagus nerve innervates the nonosseous part of the external ear canal[9]. After that, the main branch of the vagus passes into the carotid sheath, which is located between the carotid artery and the jugular vein. From there, it innervates many of the major organs. The pharyngeal branches originate from the nodose ganglia[3] and join together with pharyngeal branches from the glossopharyngeal nerve to innervate muscles of the palate, pharynx, and cervical esophagus[8]. From the nodose, the vagus runs parallel and lateral to the carotid artery. The laryngeal nerve also branches off of the nodose ganglia[3]. This nerve is closely associated with the trachea and esophagus. In the upper mediastinum, the vagus splits to innervate the bronchi, lungs, and heart[2].

The nodose ganglia houses the cell bodies of visceral afferent fibers that carry sensory information from the heart, larynx, lungs, and gastrointestinal tract to the NTS[10]. Many neurotransmitters and other neuroactive substances can effect activation of vagal afferent fibers by binding to receptors produced in cell bodies within the nodose ganglia, including glutamate, GABA, acetylcholine, serotonin, catecholamines, CGRP, tachykinins, CCK, somatostatin, galanin, NPY, endocannabinoids, and others. The neuropeptides mentioned are produced within the nodose ganglia and transported either centrally towards the NTS or peripherally towards the effector organs[10]. The neurons originating from the nodose ganglia have exhibited some form of neurochemical plasticity in relation to a number of different stimuli including food intake[10].

After the laryngeal branch branches off of the nodose ganglia, the cardiac branches of the vagus split off. The cardiac branches are composed of efferent fibers and afferent nerves from barosensors of the aortic arch[2]. Approximately twenty percent of these fibers are considered efferent[3]. The bronchial branch is made up of two or three large portions and an insignificant number of smaller filaments. About seventeen percent are efferent fibers, and of those thirty percent are myelinated and below four microns in diameter[3].

The abdominal branch is made up of approximately ninety percent sensory neurons. These portions of the vagus are made up of almost entirely unmyelinated fibers[3]. The left and right vagus nerves diverge to become the anterior and posterior trunks. There are fibers that communicate between the anterior and posterior parts of the nerve[2]. The axons tend to follow the blood vessels to the abdominal organs that they innervate[8]. About 11,000 neurons innervate the gut from the vagus nerve, eight thousand

of which are afferent[2]. The rest are considered efferent neurons, and less than one percent of them are myelinated. The anterior trunk branches into the common hepatic, ventral gastric and ventral celiac branches. The hepatic branch innervates the gastric cardia. Then, the nerve fibers divide into the gastroduodenal and pyloric branches. The gastric portion of the vagus nerve sends and receives signals from the ventral stomach and the proximal duodenum. The ventral and dorsal celiac portions come together to approach the celiac ganglion, and they innervate the small and large intestines[2]. Within the wall of the gastrointestinal tract, the vagus nerve communicates with the enteric nervous system, and it provides the small amount of control that the central nervous system has over the enteric nervous system[8].

Organ Innervation of the Vagus Nerve

The vagus innervates and affects different organs in a variety of ways[2]. Most of the sensory information that the vagus nerve carries to the central nervous system is never perceived consciously. However, in the vagus nerve, there are ten afferent fibers for every efferent fiber[11]. Some of these fibers send information from the heart to the CNS. The neurons that innervate the heart contain dense pericellular varicose endings around small autofluorescent cells. There are multifaceted terminals in the wall of the aortic arch and carotid artery and close to the glomus cells. These neurons involve mechanoreceptive and chemoreceptive functions[2].

The airways and lungs contain peptide-containing afferents that come from the jugular ganglion and innervate neuroepithelial cells[2]. Fibers from sensory neurons in the lung were present in cross sections of the cervical vagal nerve, whose branches are in the

tracheal adventitia and the bronchial adventitia, as well as the interstitium of terminal airways and alveolar sacs. The neuronal terminals are only present at specific locations in the epithelial layer of bronchi, bronchioles, terminal bronchioles, respiratory bronchioles, and alveolar ducts. These neurons branch at the epithelium. A large majority of the afferent fibers that project from the respiratory tract are C-fibers. Inhaled irritants can stimulate these neurons[12]. The cranial laryngeal nerve is involved in the cough reflex that is stimulated when substances irritate the tracheal mucosa[9]. Pulmonary neuroepithelial bodies are made up of a conglomerate of cells that produce amine and peptide neurotransmitters[12].

The vagus also innervates the pancreas and the liver. The area in which the common bile duct, the hepatic portal vein, and the hepatic artery enter the liver, known as the hilus, is the area where most of the vagal afferents are found. When they are found further away from the hilus, they are specifically located around the hepatic triads[2]. The hepatic portal vein is associated with the vagus nerve. It travels through the common hepatic branch and periarterial plexus of the common hepatic artery and interconnects with nerve fibers that innervate the portal vein. These nerve fibers end in arborizations in the adventitia and media. The bile ducts are innervated by the vagus as well, and the fibers terminate in branching varicose fibers that are in the interior and exterior walls of the bile ducts. They are located in close relation with the epithelium[2].

There are also three distinct areas where the vagus nerve fibers terminate in the stomach. These fibers, however, are outnumbered by the sensory neurons that make up the enteric nervous system[11]. These vagal fibers help the central nervous system to construct a full picture of what is going on in the gut and allow the sympathetic and

parasympathetic reflexes to coordinate regions of the gut that are far apart[11]. The external muscle layers are the first to be innervated. Collections of long, straight fibers run parallel to the muscle layer, and they are connected by oblique or right angled connecting branches. These fibers also terminate in the myenteric plexus and the lamina propria[2]. Two different vagal endings have been observed. Intraganglionic laminar endings terminate in the myenteric ganglia, and they are basket-like structures that contain tension receptors that can detect shearing forces between muscle layers. There are intramuscular arrays as well that are located in the circular and longitudinal muscle, which contain stretch receptors[1]. The intramuscular arrays run parallel to the smooth muscle nerve bundles[11]. The afferent fibers monitor ingestion through gastric distention[13]. They have low activation thresholds and achieve maximum responses within physiological levels of distention[11]. Output from these fibers is relayed to the brain via vagal and spinal sensory nerves using a variety of neurotransmitters, which will be identified later in the review[13].

The fibers that innervate the small intestine are found in the same three layers as the stomach. The area of the small intestine that fibers innervate the most densely is the duodenum. Those that originate from the myenteric plexus continue into the circular muscle layer and submucosa. These fibers form groups of branching fibers within the lamina propria of crypts and villi. The axons terminate in close proximity to the basal lamina[2].

Vagal nerve fibers have also been found in association with paraganglia, specifically in and around the glomus cells. These ganglia are located along the thoracic and abdominal branches of the vagus. Many of them are situated around the liver hilus,

following the common hepatic branch, where it splits into the celiac and gastric branches. They are often associated with nerve fascicles[2].

Central Pathways

The afferent neurons that reach the brain communicate with both the nucleus of the solitary tract (NTS) and the area postrema (AP) in the brainstem. From the AP, neurons project to the NTS and lateral parabrachial nucleus (LPBN). The NTS sends projections to the medullary motor nuclei, which includes the vagal dorsal motor nucleus, the nucleus ambiguus, motor nucleus of cranial nerve V, and rostral ventrolateral medulla (VLM), which is made up of the A1 noradrenergic group and other interneurons in the reticular formation of the brainstem. Projections from the NTS travel to the LPBN in the pons and the paraventricular nucleus (PVN), lateral hypothalamic area (LHA), arcuate nucleus (ARC), dorsomedial nucleus (DMN), amygdala, bed nucleus of the stria terminalis, and the insular cortex in the forebrain[14].

The AP is located on the dorsal surface of the medulla next to the NTS, and it protrudes into the fourth ventricle. It is made up of neurons with small diameters, astrocytes, and a few oligodendroglia. The AP is divided into sections, namely the mantle zone, the central zone, and the third zone. It is a circumventricular organ that allows for circulating signals to enter the brain without having to pass through a significant blood-brain barrier. It receives and passes on information between the blood, neurons, and cerebrospinal fluid[14]. The AP contains fenestrated capillaries, which allow circulating hormones and peptides to circumvent the blood-brain barrier[15]. It receives information from autonomic centers that are located in the medulla, pons, and the

forebrain. Efferent fibers travel to the NTS and the parabrachial nucleus (PBN), as well as to the minor A1 region of the nucleus ambiguus, dorsal motor nucleus of the vagus, and dorsal regions of the tegmental nuclei. The AP receives information from most of the same structures that it projects to. It is involved in cardiovascular control, specifically baroreceptor control. It receives information regarding the amount of sodium in circulation, as well as immune system signals[14].

Enteric Nervous System

The enteric nervous system is a part of the peripheral nervous system made up of the submucosal plexus and the myenteric plexus, and it is commonly referred to as the "brain of the gut"[16]. The submucosal plexus is partly responsible for the control of ion transport, while the myenteric plexus is involved in the control of gastrointestinal motility. The myenteric plexus is located along the whole length of the gastrointestinal tract in between the longitudinal and circular muscle layers. It is made up of intrinsic primary afferent neurons, interneurons, and motor neurons. The primary afferents are sensory cholinergic neurons. CB_1 receptors have been found on these neurons. There are both ascending and descending interneurons either directed orally or anally. The motor neurons are either excitatory or inhibitory. The excitatory neurons use acetylcholine and tachykinins to contract the smooth muscle. Inhibitory motor neurons work through the action of NO, ATP, or vasoactive intestinal polypeptide[17].

The enteric nervous system communicates directly with the central nervous system through both the vagus and the splanchnic nerves. Vagal efferent nerve fibers innervate neurons within both of the enteric plexuses. Motor axons originating from the

vagus form varicose endings that innervate almost all neurons in the myenteric plexus of the stomach and small intestine. Vagal afferents are also found innervating neurons in the enteric plexuses and within the muscle sheets. This was shown through injecting neural tracers into the nodose ganglia[18]. Therefore, the brain communicates to the enteric nervous system and the gut through vagal efferent fibers, and the gut communicates with the brain through vagal afferent fibers. This communication can have an effect on gastrointestinal secretion and motility[16].

Section III: Function of the Vagus Nerve

The vagus nerve is involved in the control of esophageal contractions, blood pressure, heart rate, and stomach and duodenal contractions. When vagal afferent fibers are stimulated, there are vago-vagal reflexes that lead to gastric emptying delay, stimulation of pancreatic secretions, attenuation of immune responses that affect inflammation, and modulation of gastric cytoprotection[19].

The vagus nerve helps to control cardiovascular and respiratory functions. The airway mucosa is innervated with C-fibers that express tachykinins and calcitonin gene-related peptide (CGRP). These C-fibers respond to capsaicin. This response to capsaicin is activated through the vanilloid receptor-1 (VR_1). The neurons help to control airway functions when the body must deal with irritants that have been inhaled. Stimulation of the vagus nerve fibers causes bronchoconstriction, mucus secretion, and shallow breathing. Pulmonary reflexes can also induce apnea, bradychardia, and hypotension[20].

Capsaicin-sensitive lung vagal afferents are implicated in the control of respiratory functions in both normal and pathological conditions. They are excited by chemicals and irritants, which cause cough and constriction of bronchioles. Bradykinin, histamine, prostaglandin E2, adenosine, and epinephrine are compounds that increase excitability of these afferent fibers. VR_1 receptors respond to changes in heat, pH, capsaicin, endocannabinoids, and arachidonic acid. The endocannabinoid anandamide (AEA) is implicated in a variety of inflammatory conditions, and it causes a concentration-dependent increase in excitability in these capsaicin-sensitive fibers[21].

The vagus nerve and the brain regions that it communicates with are highly involved in control of food intake. The AP is very important in the process of conditioned flavor learning and aversions. The AP and NTS are innervated by abdominal vagal afferent fibers. These afferents respond to gastric distension and chemical stimuli[22]. These areas of the hindbrain are also important in the consumption of highly palatable foods, as was demonstrated by an experiment by Edwards and Ritter in 1981 involving AP lesions and preferred foods. They found that lesions of the AP were responsible for causing an increase in intake of preferred foods without decreasing the amount of normal food or water consumed. This response may result from loss of signals received by other parts of the brain, such as the NTS. The rats that received the lesions still responded to molecules like cholecystokinin (CCK) and gastric preloads. A chemoreceptor in the AP may influence food intake by affecting the information that arrives from taste afferents in the rostral NTS[15]. Overingestion of palatable foods by rats that have had AP lesions is not due to diminished action of the vagus nerve or decreased

response to satiation cues like CCK. Therefore, it is due to different responses to the quality of the food as opposed to solely the quantity[22].

Cardiac and pulmonary vagal afferents terminate in the lateral subnuclei of the NTS. Signals from the gastrointestinal tract terminate in the medial subnuclei, AP, and the dorsal motor nucleus. Neurons in the AP and the NTS further project to the LPBN in rats. Lesions of the LPBN reverse the overingestive effect that AP lesions induce in rats [21]. It may be that the nerve fibers that are destroyed by AP lesions affect the activity of LPBN nerve fibers that integrate and send information to the forebrain, which is responsible for sensory, gustatory, and chemosensory information[23].

The current viewpoint on the role of the hypothalamus regarding food intake is that it contains neuronal circuits as opposed to centers. Signals sent from the periphery are integrated to affect food intake and the amount of energy used. Therefore, the control of body weight involves hypothalamic circuits that have been well studied. It contains cell bodies and axons that process information from the peripheral, hormonal, and central sources. There are several nuclei in the hypothalamus important to visceral controls. These nuclei interface with each other as well as with areas of the brainstem[24].

The ARC of the hypothalamus is important in the control of food intake. It is located above the median eminence. It is also considered a circumventricular organ by some researchers[25]. One group of nerve fibers expresses both agouti-related peptide (AGRP) and neuropeptide Y (NPY), and they are involved in increasing food intake. Another group expresses melanocyte-stimulating hormone (MSH) which can decrease the amount of food eaten[26]. These neurons are also sensitive to leptin and insulin. These signals will be further explored later in the review. Both of these groups of fibers project

to the PVN, ventromedial nucleus (VMN), dorsomedial nucleus (DMN), and LHA of the hypothalamus as well as the dorsal vagal complex including the NTS and dorsal medullary ventricle (DMV)[27]. When adiposity signals are sent to the arcuate nucleus, it responds by secreting anorexigenic signals[26]. The PVN is influenced by concentrations of leptin, orexin, CCK, and glucagon-like peptide-1 (GLP-1). The neurons in this nucleus express receptors for corticotrophin-releasing hormone (CRH) and thyrotropin-releasing hormone (TRH). These signals are involved in the control of food intake as well as control of the hypothalamus-pituitary-adrenal axis and the thyroid. The PVN also projects directly to the NTS and the AP. This is a reciprocal path as the NTS sends signals to the PVN directly, while AP information is indirect[27]. The VMH contains neurons that respond to distention of the stomach. They are also responsible for glucose homeostasis. It was once considered the "classic satiety center." The DMN of the hypothalamus contains neurons that express NPY. These neurons communicate with the ventromedial hypothalamic nucleus as well as the lateral hypothalamus. It integrates and processes information from those areas. The LHA was once considered the "feeding center"[25]. It contains nerve fibers that are inhibited by leptin and contain glucose receptors. There are also many fibers that express orexigenic signals. These are activated by starvation, hypoglycemia, and low glucose in the brain[27].

Hunger is defined as a strong desire or need for food, while satiety is described as the condition of being full or gratified[27]. Appetite involves the frequency and amount eaten during a meal, what foods are eaten, the energy density, variety, and palatability[25]. The old theory on how appetite and satiation occurs involves the LHA, which measured the fall of glucose in the circulation and increased food intake. Then, the increase in

blood glucose after a meal activated the VMH, or the satiety center, which then inhibited the LHA, forming a sort of negative feedback loop. However, it has been found that chemicals only affect the onset of a meal when energy is extremely low[26]. Now it is known that the hindbrain is a major site that receives signals that trigger satiation, based on lesion studies. The vagal afferents project to the NTS, and some peptides effect the AP directly, such as amylin[24]. Pregastric, gastric, and intestinal signals that trigger satiety can be combined and function at the same time[13].

The stomach may monitor the volume ingested. However, distention of the stomach is not the only signal necessary to trigger the termination of ingestion[13]. Bayliss and Starling discovered secretin, one of the first gut hormones[28]. It is released by luminal acid. Enteroendocrine cells relay information on luminal contents that secrete signals at the basolateral side of the cell. These signals aid in control of digestion and food intake. They also regulate nutrient delivery. The targets of these signals include secretory cells, smooth muscle, proliferating cells, and brain signaling to determine the amount of food eaten. The neurotransmitters and signals involved within the brain will be described later in the review. The second pathway of signaling is through subepithelial nerve fibers that also respond to signals in the stomach lumen that cross through the epithelium, including short-chain fatty acids[29].

The intestine can send signals that slow gastric emptying, which decreases the amount of fluid entering the small intestine. Peptides that act as hormones can be released from enteroendocrine cells in the small intestine to induce satiation[29]. These signals activate vagal and sympathetic afferents that terminate in the NTS[26].

Sensory neurons involved in satiation are considered intrinsic primary afferents. The gut wall houses the cell bodies. These neurons are involved in the beginning of reflex responses, like the mixing and propulsive movements, changes in the flow of blood, and water and electrolyte secretion. These fibers respond to changes in the lumen, distention, and mechanical distortion of the intestine. Dogiel type II neurons involved in the intestine react to inorganic acids and fatty acids. These neurons have a multipolar phenotype that contains branches in the lamina propria of the mucosa. There are also axons that head into the myenteric ganglia. The submucosal ganglia also house the cell bodies for mucosal mechanoreceptors[30].

Other sensory neurons are considered extrinsic primary afferents. These fibers have cell bodies housed in the vagal and dorsal root ganglia. They send information about the gut to the central nervous system. They are involved in regulation of all aspects of gastrointestinal function, including the visceral sensation of "pain". The axons whose cell bodies are in the nodose ganglia travel through the vagus nerve. These afferents react to mechanical probing and also to chemicals infused into the lumen. There are mechanoreceptors in these areas that have both low and high thresholds. Those with low thresholds react to normal tissue states during the course of digestion, while those with high thresholds respond to pathophysiological states. Intestinofugal neurons that contain their cell bodies in the gut and terminate at neurons outside of the gut wall are another example of sensory neurons in the gut. These form synapses with pre-vertebral sympathetic ganglia that project back to the gut. They are contained mostly in the colon. These affect movement and act on orad sites[30].

Food causes enteroendocrine cells to release peptide hormones that move across the lamina propria to excite afferent nerve fibers or to enter the bloodstream. Another way for communication involves uptake and metabolism of glucose, which causes secretion of peptides through potassium channel closure, depolarization, and calcium channel activation[13].

The vagus also has anti-inflammatory action by working through an efferent cholinergic pathway. The nerve fibers suppress inflammatory responses by releasing acetylcholine. Animals that have had their vagus nerves removed are more sensitive to endotoxin and produce more tumor necrosis factor-alpha (TNF-α). When these fibers are stimulated, it activates the pathway which causes inhibition of cytokine release. This triggers an interaction between the α-7 subunit of the nicotinic receptor on macrophages, and it inhibits the activation of macrophages, leading to an anti-inflammatory affect. NF-κB, a transcription factor that is involved in the expression of pro-inflammatory proteins, increases in expression after the vagus nerve is removed. This is correlated with an increase in inflammation in the gut. The anti-inflammatory action is affected by the nicotinic acetylcholine receptor expressed by macrophages and endothelial cells during sepsis and inflammation. The vagus also suppresses lymphocyte activation[31].

The vagus has an effect on immune function, which in turn has an effect on ingestive behaviors. Pathophysiological states decrease food intake by having an effect on basal forebrain and diencephalic neurocircuitry that integrate noxious or aversive stimuli with that which controls feeding behavior. This response may be through a danger channel or through the increase of ascending projections that carry satiation signals, or both. The danger pathway would change the pattern of activated neurons in

the brainstem after immune challenge. The neurons involved in the danger pathway project to the PVN, which is involved in the coordination of autonomic and neuroendocrine responses to the physiological challenge. If it works through the same pathway as satiation, then the same groups of nerve fibers should be activated regardless of the type of stimulus that causes the activation. Gaykema performed experiments to determine which of these pathways are responsible for the immune response using lipopolysaccharide (LPS) to cause colitis[32]. Immune challenge inhibits ingestion through both danger and satiety related pathways. TNF-α is an illness-induced signal that has an effect on brainstem mediated responses to pathophysiological conditions and causes the activation of danger-related projections that work through the VLM, NTS, and the external lateral parabrachial subnucleus[32]. The vagus nerve also contributes to the feelings of "pain", such as scratching, tearing, and burning sensations. These fibers terminate in the paratrigeminal nucleus[1].

Section IV: Sensation

The nodose ganglia of the vagus nerve have been found to contain receptors for glutamate, catecholamines, serotonin (5-HT), acetylcholine, substance P, neurokinin A, vasoactive intestinal peptide, CGRP, somatostatin, CCK, cannabinoids, as well as other neurotransmitters[33]. Receptors that are expressed in the nodose ganglia are transported to the central and peripheral terminals. The ligands of these receptors may circulate through the blood or through paracrine systems[22]. The vagus can also be responsible for transmitting mechanosensory signals to the brain. It can monitor the stretching and

contracting of smooth muscle. For example, mucosal touch receptors have a low threshold and adapt rapidly. Muscular tension receptors are activated by contractions that are spontaneous by the external muscle layer stretching. There are also serosal mechanoreceptors that adapt slowly[1].

The hormones or paracrine factors secreted by the gastrointestinal tract are secreted in response to nutrients in the lumen. These hormones or paracrine factors may activate vagal afferents. It has been proposed that afferents use glutamate as a primary transmitter for excitation. Vagal afferents also use gamma-aminobutyric acid (GABA) as an inhibitory neurotransmitter[19].

Anorexigenic Neuropeptides- Central Action

Glucagon-Like Peptide

Glucagon-like peptide-1 (GLP-1) is synthesized by the cleavage of proglucagon[13]. Preproglucagon is expressed in α cells in the pancreas, L cells in the gastrointestinal tract, and neurons in the NTS. When this zymogen is cleaved, it can create glicentin, oxyntomodulin, GLP-1, or GLP-2[25]. It is expressed in the gut, pancreas, and the brain. It is generally found in neurons that also express oxyntomodulin and peptide YY. Its secretion is caused by the ingestion of fats and carbohydrates due to neurohumoral mechanisms. It is broken down and deactivated by dipeptidyl peptidase 4. Some of its actions include releasing glucose-dependent insulin, inhibiting glucagon secretion, and increasing β cell growth in the pancreas[13]. It can decrease body weight by decreasing the size of a meal and increasing thermogenesis and energy expenditure[27]. It may delay gastric emptying; however, its short half-life stops it from being a useful treatment for

obesity[26]. The receptor GLP-1R is found in the gut, pancreas, brainstem, hypothalamus, and vagal afferents. The peptide has been found to cross the blood-brain barrier[13]. GLP-1 is implicated in leptin's effects on hunger and satiety and may be one of the targets for leptin's anorexigenic effects. The GLP-1 receptor and GLP containing nerve fibers are generally found in the hypothalamic midline nuclei, and its effect is communicated through NPY or leptin. GLP-2 is mainly found in the DMH, and it effects behavior[25]. Therefore, GLP acts on GLP-1 receptors to decrease the amount eaten during a meal and increase energy expenditure through action on nerves that communicate with hypothalamic midline nuclei.

Cocaine-and-Amphetamine Related Transcript

Cocaine-and-amphetamine regulated transcript (CART) has an anorexigenic effect on the body that is partly in response to the release of GLP-1[26]. It is a neuropeptide that is found in areas of the hypothalamus including the PVN, DMN, perifornical regions, lateral nucleus, and the arcuate nucleus. There are many aspects of physiology that CART influences, including reinforcement and reward, sensory processing, stress, endocrine regulation, and feeding. The regulation of CART mRNA involves energy availability[25]. It is typically co-expressed in vagal afferent neurons that also contain CCK-1 receptors. CCK has been shown to regulate the expression of CART in vagal afferents, and it depends on protein kinase C (PKC) and CREB for its effects[34]. CCK can behave like a switch that stimulates the expression of CART. Whether and how much CART or melanin-concentrating hormone (MCH) is expressed depends on the energy availability throughout the day[34]. In conclusion, CART has an anorexigenic effect on the body based on energy availability, and its expression is mediated by CCK.

Oxyntomodulin

Oxyntomodulin is one of the products that are released from the cleavage of preproglucagon. It is secreted from distal intestinal L cells proportionally to the amount of calories taken in. It is used to decrease the amount of a meal and increases the amount of energy used. It probably has its action through GLP-1R, and the neurons that it acts on are mainly located in the hypothalamus[13].

Pro-opiomelanocortin

Pro-opiomelanocortin (POMC) is a molecule that is the precursor to several others, one of which is MSH. It acts on MC3 and MC4 receptors that can be found in the ARC[26]. The melanocortins are considered bioactive peptides. POMC is found in the anterior and intermediate pituitary, skin, the immune system, and hypothalamic neurons. Leptin enhances the release of melanocortins, which leads to the possibility of a positive feedback loop that relates sites of melanocortin release and adipose tissue release of leptin[25]. Therefore, POMC aids in the anorexigenic effect that leptin has on the body.

Corticotrophin Releasing Hormone

Another hormone that affects food intake is corticotrophin releasing hormone (CRH). This hormone is a 41-amino acid neurohormone that regulates the secretion of adrenocorticotropic hormone (ACTH) by the pituitary[23]. CRH is expressed in the PVN, and its expression is involved in increasing stress-related endocrine, autonomic, and behavioral responses[27]. It affects caloric intake without the presence of stress by inhibiting appetite. There are two receptors that the hormone acts on, CRH-1 and CRH-2. It has specific effects based on the area at which it acts. Leptin has an inhibitory effect on CRH expression in the PVN. Therefore, leptin decreases the effect that CRH

typically has on the activity involved in the hypothalamus-pituitary-adrenal axis. However, leptin also increases expression of CRH-2 in the VMH nucleus[25]. CRH is involved in inhibiting food intake and reducing body weight[27].

Neurotensin

Neurotensin is a peptide that is made up of thirteen amino acids. There are three receptors that this peptide binds to, including NTRL (low-affinity), NTRH (high-affinity), and NTR. They are G-protein coupled receptors that regulate cAMP, cGMP, and phosphoinositol turnover, calcium influx, phospholipase C (PLC), and sodium-potassium ATPase activity. This peptide is produced in the ARC, PVN, and DMH. It may be involved in the effects of leptin to decrease appetite and food intake[25].

Anorexigenic Neuropeptides- Peripheral Action

Cholecystokinin

CCK-8 immunoreactivity has been found in the cell bodies, fibers, and fiber terminals of the vagus nerve[2]. It is produced by I cells in the duodenal and jejunal mucosa and in the brain and enteric nervous systems. It acts on the gut through neural pathways. The prepropeptide is processed by endoproteolytic cleavage to produce at least six different peptides. These different forms have a carboxy-terminal octapeptide with an oxygen-sulfated tyrosine in common[13].

CCK is released due to depolarization of the cell body. The depolarization is caused by calcium ion influx[2]. This increase in calcium concentration can be inhibited by calcium channel blockers[29]. Fatty acids that contain more than twelve carbons cause CCK release[6]. CCK-8 neurotransmitter activity has been established moving toward the

gut in the vagus nerve in dogs and cats by Dockray in 1981 through experiments involving vagal ligation and radioimmunoassay methods[2].

CCK causes the induction of satiety[2]. It limits meal tolerance by delaying the emptying of the stomach[35]. It helps match the amount of liquid delivered to the duodenum with the ability for digestion. This occurs by stimulating secretions from the pancreas and the contraction of the gall bladder, so that enzymes and bile salts are appropriately delivered. Stimuli that effect CCK include gastric distention, leptin, interleukin-1-β (IL-1β), urocortin, and apolipoprotein IV[19].

Lal et. al. (2004) performed an experiment involving human subjects that was used to demonstrate that C_{12} fatty acids reduce the volume of contents delivered into the duodenum more than a C_{10} fatty acid. It also showed that the volume of contents in the stomach is the main cause for the tolerance limitation. The feeling of satiation continued for a longer period after C_{12}, due to the slowing of gastric emptying. The cause for this decrease in volume delivered to the duodenum may include many factors, including reduced gastric tone, reduced peristalsis, or increased intestinal tone[35]. When CCK is given right before a meal, the amount eaten decreases in a dose-dependent way without affecting the amount of liquid consumed or causing nausea. This action does not last very long[13].

There are two different known receptors for CCK. The CCK-1 receptor is available mostly in the gastrointestinal tract. It is found primarily on vagal afferents with a relatively small diameter that innervate the stomach, jejunum, and the hindbrain. The administration of the ligand yields increases in vagal afferent firing in the hindbrain region. The afferents that have this receptor often also express receptors for leptin. The

receptors are from regions of the body that are innervated by the gastric, celiac, and hepatic branches of the vagus nerve[13]. Low and high-affinity CCK-1 receptors are both found in the nodose ganglia. When they are activated at different states, different actions may result. Activation of PKC may phosphorylate serine or threonine on CCK-1 receptors. This leads to desensitization. PKC activated by high-dose CCK inhibits the activity of high-affinity CCK-1. The CCK-2 receptor is primarily found in the brain, specifically in the hypothalamus and the hippocampus[36].

The CCK-1 receptor is a G-protein coupled receptor, and there are a variety of signal transduction pathways that are activated through CCK binding to the CCK-1 receptor. Activation of the CCK-1 receptor can cause hydrolysis of phosphatidylinositol bisphosphate by phospholipase C to produce inositol triphosphate and diacylglycerol, which causes the release of calcium and stimulates protein kinase C[37]. In addition, CCK can activate adenylyl cyclase, leading to an increase in cAMP concentration and activation of PKA. There have been other studies that have linked CCK-1 receptor activation with p38-MAPK signal transduction cascades[37]. Another pathway of interest induced by CCK-1 receptor activation involves the activation of IκB kinase, which phosphorylates IκB, allowing NF-κB to be translocated into the nucleus and leading to the production of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6[37].

In conclusion, CCK binds to CCK-1 or CCK-2 receptors in the gastrointestinal tract or the brain, respectively, to delay gastric emptying and limit meal tolerance based on fatty acid ingestion.

Leptin

Leptin is considered a long acting adiposity hormone that works on the central and peripheral nervous systems[13]. It is produced in white and brown adipose tissue, the stomach, placenta, mammary glands, ovarian follicles, and fetal organs[25]. This hormone acts in the brain, particularly in the hypothalamus, to increase central nervous system sensitivity to signals from other short-acting peripheral satiation signals. The specific areas of the hypothalamus that are affected include the arcuate nucleus, the PVN, the DMV, and the LHA[13]. Leptin has been found to be capable of crossing the blood-brain barrier, which aids in its ability to activate hypothalamic pathways for satiety and energy expenditure.

More than one isoform of leptin is found in a majority of tissues[25]. The receptors for leptin are located on specific L-cells located in the distal small intestine and colon[13]. Ob-Ra is the short form that is primarily expressed in the choroid plexus and brain vessels. It is involved in the transportation of leptin from the circulation into the central nervous system[38]. Ob-Rb is the long form and is responsible for the appetite suppressing action. Leptin stimulates a signaling pathway that causes the inhibition of orexigenic peptides, such as NPY, MCH, orexin, and AGRP, while stimulating anorexigenic ones, for example MSH, CART, and CRH. Expression generally follows a circadian pattern[25].

Leptin is expressed by gastric chief cells and adipocytes[29]. The concentration of leptin that circulates is proportional to the amount of adipose tissue in the body by mass, and it typically increases after a meal has been ingested[27]. It decreases meal intake through its effects on the release of other anorexigenic and orexigenic peptides. Some

forms of obesity may be a result of leptin resistance, or obesity may result in leptin resistance.

Peptide YY and PP

The PP-fold family of peptides, which includes neuropeptide Y (NPY), peptide YY (PYY), and PP, contains an amidated C-terminal. They interact with receptors that involve inhibitory G-proteins. PYY is made by distal intestinal I cells[26]. It takes action on the gut through neurological pathways[16]. It is secreted after ingestion of a meal proportionally to the amount of calories that have been ingested, similar to leptin. Lipids effect secretion more than carbohydrates do, and carbohydrates are more effective than proteins. Its secretion is stimulated by atropine sensitive neurons in the foregut as well as nutrient stimulation in the hindgut. It is broken down by the enzyme dipeptidyl peptidase-4 (DPP4), and this produces another active peptide. PYY acts at the hypothalamus via afferents to the NTS[26]. It is responsible for decreasing hunger and meal intake. It slows the emptying of the stomach, causes secretions from the stomach and the pancreas, and increases fluid and electrolyte absorption. It facilitates action potential frequency in POMC neurons in the ARC and inhibits glutamatergic transmission between the NTS and DMV[26]. It acts on Y receptors, which are G-protein coupled receptors that are activated by members of the PP-fold family including NPY, PYY, and PP. The lack of PYY has been shown to be correlated to obesity[25].

There are two major forms, PYY1-36 and PYY3-36. PYY3-36 acts mainly on Y2 receptors. It travels through the circulation after a meal, and the concentration is proportional to the amount ingested. When it acts on Y2, NPY neuronal action is inhibited. Its effect on appetite may depend on making environmental stress minimal[25].

PP is another member of the PP-fold family. It is produced in islet cells, and it is secreted after a meal proportionally to the amount of calories that were ingested. It acts on Y4 and Y5 receptors. Its actions influence biliary and exocrine pancreatic function, gastric acid secretion, and gut motility. It generally decreases the intake of calories via Y4 in the AP and when it is present centrally, it increases food intake through Y5 receptors in the PVN of the hypothalamus[13].

Therefore, the members of the PP-fold family that are anorexigenic, PYY and PP, are secreted in proportion to the amount of calories ingested and act on Y receptors to slow stomach emptying and increase absorption.

Insulin

Insulin is produced by the pancreas so that the concentration increases when the body is in a state of positive energy balance[25]. It is considered an adiposity signal that is involved in energy homeostasis. The levels in circulation elevate immediately following a meal. There are receptors located in the ARC, VMH, PVN, AP, NTS, and DMV. The receptors are present on the same neurons as MSH and NPY in the ARC[27]. It can cross the blood-brain barrier, and the concentration of insulin present in the brain is proportional to the amount in the blood stream[26]. It crosses the barrier through a receptor-mediated process. This process involves an extracellular β subunit that binds to insulin and another intracellular subunit that passes the signal along. The intracellular subunit has intrinsic tyrosine kinase activity. Insulin receptor substrate-1 (IRS-1) and IRS-2 have been found on neurons[25]. These receptors are responsible for the reduction of energy intake. Insulin deficiency has been associated with increased NPY[26].

Amylin

Amylin is a 36 amino acid long peptide which is cosecreted with insulin by pancreatic β cells. It is involved in decreasing gastric emptying and secreting gastric acid and glucagon. Due to these actions, it decreases the amount of food eaten at a given meal. It acts primarily on the AP[13]. The effect could be in relation to the reduced expression of orexigenic peptides in LHA or increased expression of POMC in the ARC[27]. The effect of amylin is independent of the vagus nerve[39]. It has an effect on the actions of CCK, glucagon, and bombesin[25].

Neuromedin B and Gastrin-Releasing Peptide

Bombesin is a peptide made up of fourteen amino acids derived from frog skin. There are a couple of known peptides related to bombesin, including neuromedin B and gastrin-releasing peptide. These peptide molecules bind to G-protein coupled receptors and cause responses through the action of PLC. Receptors for these peptides are located in the gastrointestinal tract and the hypothalamus. The production and secretion are induced by distention of the GI tract. It is another messenger that informs the nervous system of gastrointestinal filling[25].

Enterostatin

Enterostatin is another anorexigenic peptide whose production is stimulated by the ingestion of fatty acids. It is a pentapeptide that is produced from the cleavage of prolipase, which is secreted from the exocrine portion of the pancreas. It is also produced in the gastrointestinal tract and the brain. It acts to decrease dietary fatty acid consumption. It achieves this action through mechanisms that may involve the F1-ATPase β subunit[13].

Apolipoprotein

Apolipoprotein A-IV is a glycoprotein that is secreted by the intestine after fat absorption and the formation of chylomicrons. It aids in the packaging of lipids for movement through the lymphatic system to the blood. The glycoprotein is also made in the ARC of the hypothalamus. This protein is responsible for decreasing the size of a meal and increasing weight in rats[13].

Serotonin

Chemical and other types of stimuli cause 5-HT to be released from enterochromaffin cells that make up the lining of the small intestine. This activates 5-HT_3 receptors which cause pancreatic secretion. This molecule is also produced in the raphe nuclei of the midbrain, and it acts on the PVN, VMH, and suprachiasmatic nuclei[25]. The stimuli that result in its release include changes in osmolarity and products of digestion of carbohydrates. Significant nodose responses result from these stimuli, due to the 5-HT release which causes excitation of signal transduction pathways. Osmolarity and digestion products stimulate 5-HT secretion, which act on receptors located on vagal afferent fibers to stimulate them, as shown in an experiment done by Wu. This experiment involved cannulating a Sprague-Dawley rat's duodenum and perfusing different test solutions when the vagus was both intact and cut. These experiments showed that glutamate may serve an important role in the signal transduction pathways activated by 5-HT. Substance P may also play a critical role in those pathways. The neurons that respond to 5-HT play a role in the vago-vagal reflex caused by stimuli in the lumen[33].

Serotonin also has an effect on emesis[6]. It diminishes food intake and weight by reducing appetite and increasing energy use[26]. It is specifically involved in meal satiation[25]. The receptor is a ligand-gated ion channel that has been implicated in mood and pain. Cannabinoids inhibit serotonin's release in a dose-dependent manner; however, the effects of this molecule are not mediated through cannabinoid receptors. Cannabinoids may act allosterically at another site on the receptor[40]. Serotonin has an anorexigenic effect on food intake through action on 5-HT_3 receptors on neurons that originate in the dorsal raphe nucleus of the midbrain and project to nuclei of the hypothalamus[25].

Orexigenic Molecules- Central Action

Neuropeptide Y

Hypothalamic NPY is considered one of the most powerful enhancers of appetite. It is made up of 36 amino acids, and both the N-terminal and the C-terminal are tyrosines[25]. The neurons in the ARC that express NPY also express AGRP in 90% of cases. It works to inhibit thermogenesis while it increases the amount of food ingested and increases adipogenesis[26]. It has been shown to have influences on many body functions, including ingestion, cardiovascular regulation, and control of neuroendocrine axes, affective disorders, seizures, and memory retention. It is expressed mainly in the ARC, and the neurons project to the PVN, DMH, and LHA. There are five receptors that it acts on that are G-protein coupled, including Y1, Y2, Y4, Y5, and Y6. These receptors act by decreasing adenylate cyclase and cAMP while increasing calcium intracellularly. The synthesis and release of NPY are regulated by signals involving leptin, insulin, and

glucocorticoids. More NPY is produced when energy is deficient or when more energy is needed. It is believed that the primary role may be to maintain energy homeostasis and body fat while the body is under energy deficient conditions[25]. NPY seems to specifically increase the intake of carbohydrates. In an experiment done by Edwards et. al.[40], the AP and NTS of Sprague-Dawley rats were lesioned and animals were tested for the intake of palatable foods. Once they had been euthanized, the ARC, PVN, VMH, and LH were analyzed for NPY by radioimmunoassay. The results showed that those with AP lesions have elevated levels of NPY in the hypothalamus. The mRNA of NPY is elevated specifically in the basomedial hypothalamus, while the peptide is increased in the PVN. This increase in NPY concentration may be implicated in the increased appetite for highly palatable foods. NPY is implicated in maintenance of energy homeostasis by interacting with Y receptors on neurons that project to the hypothalamus. Basically, it is a hormone that is present both in the brain and the gut, and it takes action through vagal pathways[40].

Galanin

Galanin is a neuropeptide that is made up of 29 amino acids. The peptide is amidated at the C-terminal. It is found both in the brain and in the gut. Its actions effect cognition, memory, sensory and pain processing, neurotransmitter and hormone secretion, and feeding behavior[25]. The peptide works through G-protein coupled receptors and ion channels. Specifically, it inhibits gastric neuropeptides through its effects on potassium ion channels. These actions mimic those that GABA and NPY have on neurons[25]. The receptors tend to be on the same cells as those for GABA, norepinephrine, 5-HT, and NPY in several different regions of the brain. Galanergic

neurons terminate in the NTS[25]. Galanin is responsible for an increase in the consumption of fatty acids. Galanin-like peptide (GALT) has been shown to effect body weight, adiposity, and reproductive function. This peptide is produced in the ARC and projects to the anterior PVN. These neurons also contain leptin receptors[25].

Melanin-Concentrating Hormone

Melanin-concentrating hormone (MCH) is a cyclic peptide that is made up of nineteen amino acids which are cleaved from prepro-MCH. The neurons that utilize this neuropeptide are mostly located in the lateral hypothalamus and zona incerta[25]. It causes hyperphagia along with a gain in body weight[26]. It acts through two different receptors, which are both G-protein coupled receptors, MCH-1R and MCH-2R. These receptors are located in many parts of the brain, including the hippocampus, amygdala, and cerebral cortex, as well as on the nodose ganglia of the vagus nerve. It also plays a part in arousal when it has to do with goal oriented behavior such as eating[25]. The concentration of MCH increases in the lateral hypothalamus when a rat has been fasted. MCH is also found in the intestine, in accordance with results shown by Burdyga[41], which implies that it is involved in peripheral and central control of appetite. MCH and MCH-1R are both produced in the same nerve populations, and their expression is related to fasting. This seems to imply that there is a positive feedback loop regulating the production of both the neuropeptide and its receptor. The expression is increased as fasting continues. Administration of CCK seems to effect the concentration of MCH and its receptor in the body, implying that they work synergistically to provide signals for satiation[41]. Therefore, MCH is implicated as a player in food intake after fasting.

Agouti-Related Peptide

Agouti-related peptide (AGRP) is released by the ARC[26]. It is a peptide made up of 132 amino acids[25]. The concentration of AGRP is decreased by leptin. The expression is inversely related to that of MCH. It is usually secreted in response to a lack of energy balance in the body[26]. It has a role in regulating feeding and weight. All of the neurons that contain AGRP also secrete NPY, and they terminate at the hypothalamus and other sites. It is an antagonist of melanocortin receptors[25]. AGRP increases food intake to maintain energy homeostasis.

Orexin

Orexin has been found in the hypothalamus and in gastrointestinal endocrine cells[29]. There are two different forms, orexin-A (OXA) and orexin-B (OXB). OXA is made up of thirty-three amino acids, and OXB is made up of twenty-eight. There are many orexin receptors in the central nervous system. Orexin-R1 is found on vagal afferent nerve fibers as well as in the ARC, VMH, and suprachiasmatic nucleus (SCN). OX-R2 is found mostly in the PVN[25]. These are G-protein coupled receptors. OXA has been found to inhibit the effects of CCK[29]. The way that the body interacts with OXA may be affected by circadian rhythms[26]. It causes an increase in calcium concentration within the cell by activating PKC. It has orexigenic effects by inhibiting the feeling of satiation. This signal is expressed when the body is in a nutritional deficiency. Neurons that express the receptors are regulated by signals such as leptin, glucose, and ghrelin[25].

Orexigenic- Peripheral Action

Ghrelin

Ghrelin is made up of 28 amino acids, and it is made in endocrine cells in the gastric mucosa[27]. It is secreted by the A-X cells of the oxyntic glands of the stomach. The peptide is acylated in the stomach and proximal small intestine. It can also be found in the placenta, kidney, heart, thyroid, and Leydig cells[26]. The peptide causes an increase in food intake. It also increases gut motility, and it decreases the secretion of insulin. The amount of ghrelin in circulation increases before meals and decreases when food is eaten[26].

The receptor for ghrelin is typically located on vagal afferent neurons or on neurons of the hypothalamus-pituitary circuit on neurons that also express NPY and GHRH[26]. The receptor is G-protein coupled. The neurons that express ghrelin receptors are also known to contain receptors for CB_1 and MCH1. Ghrelin receptor expression does not increase when challenged with energy restriction. Ghrelin also causes a decrease in CCK receptor activation[42]. It has its effect on the hypothalamus, caudal brainstem, and mesolimbic reward centers. It acts synergistically with NPY and AGRP in the ARC[27]. Ghrelin also plays a part in the control of ACTH and prolactin secretion, glucose and lipid metabolism, gastric motility and acid secretion, heart function, sleep, and reproduction. Administration of ghrelin causes an increase in the mass of fat in the body, because it inhibits lipid oxidation and increases food intake through action on receptors on vagal afferent neurons that innervate the hypothalamus and brainstem[26]. This hormone is found both in the brain and the gut, and it affects the gastrointestinal tract through neural pathways[16].

Chemical Relationships in Vagal Afferent Neurons

The specific phenotype of the vagus nerve depends on the caloric intake through the day[19]. The CB_1 and MCH-1 receptors are expressed more in a state of caloric deficiency, while Y2 receptor concentration decreases in the same state. Specifically, CB_1 receptors are maximally expressed after a full day of fasting. Once one eats, there is a rapid decrease in CB_1 expression after two and five hours. The expression is inhibited by CCK[43]. Anandamide, an endocannabinoid, is increased in the small intestine in a fasting state. Vagal afferents tend to express both MCH-1 and CCK-1 receptors[42]. Y2 receptor expression is increased with CCK expression. Different populations of neurons mediate PYY effects on food intake and emptying of the gastrointestinal tract[44].

Leptin and CCK have been shown to work together in the control of satiation and body weight by acting on vagal afferent neurons. Most vagal afferents that have receptors for leptin also contain receptors for CCK. Experiments done by Peters have shown that these two stimuli may act synergistically[45]. CCK may play a part in the regulation of leptin's access to the brain targets, including the hypothalamus[38]. In Merino's study involving the administration of CCK-8 and leptin, it was discovered that CCK-8 attenuates plasma leptin while it increases its concentration in the cerebrospinal fluid; therefore, "treatment with CCK during periods of positive energy balance activates metabolic processes leading to a decrease in body weight." CCK-8 may increase the uptake of peripheral leptin into hypothalamic areas[38].

The effect of CART on the decrease of food intake is prolonged by the administration of CCK. In other words, the expression of CART in the nodose ganglia was attenuated by food withdrawal and the expression returned to the original state when

food was ingested. Therefore, CCK release following food intake is responsible for the synthesis of either an orexigenic or anorexic peptide depending on the energy state of the individual[19].

Ghrelin also has an effect on the synthesis and expression of specific receptors on vagal afferents[19]. When it has been injected before food was reintroduced, it reversed the attenuation of expression of the CB_1, MCH, and MCH-1 receptors and it increased CART and Y2 receptor expression[19].

CCK and 5-HT have both individual actions and those that work synergistically. They may stimulate vagal afferents using either paracrine or endocrine transmission. Together, they are responsible for a majority of pancreatic enzyme secretion. Some vagal afferents express both CCK-1 receptors and $5\text{-}HT_3$ receptors. The interaction may add to the maximal effect that CCK has on satiation. This may explain why many patients with irritable bowel syndrome show sensitivity to CCK[36].

CCK also interacts with PYY, according to studies by Burdyga in 2008, which quantitatively analyzed Y2 and CCK-1 receptor expression through immunohistochemistry after CCK, PYY, or saline injection interperitoneally. The results have shown that CCK stimulates Y2 receptor expression in vagal afferent neurons that innervate the stomach. This occurs through PKC activation. Those neurons that innervate the ileum and colon, on the other hand, do not seem to be regulated by CCK[44].

The purpose of reviewing the many different satietogenic hormones involved in feeding behavior is to point out that no single molecule controls appetite and feeding behavior. The aim of this study and any future studies involving these hormones must

focus on how these molecules interact to impact appetite, feeding behavior, and gastrointestinal motility.

Section V: Cannabinoids

Cannabinoids have historically been used in the treatment of gastrointestinal pain, flatulence, gastroenteritis, Crohn's disease, diarrhea, and diabetic gastroparesis. Endocannabinoids can be synthesized and secreted based on the body's demand[46]. They are defined as those endogenous compounds that act on cannabinoid receptors and other synthetic compounds that are chemically related. This includes THC as a prototype as well as drugs that have been synthesized, such as CP-55,940 and WIN55,212-2. Endogenous cannabinoids include anandamide (AEA) and 2-arachidonoyl-glycerol (2-AG)[47].

Endocannabinoids are produced in response to depolarization of post-synaptic neurons, and they diffuse through the plasma membrane into the synapse. From there, they bind to receptors on pre-synaptic or neighboring cells. These receptors are coupled to G-proteins. They have the typical G-protein coupled receptor morphology, complete with the seven-transmembrane region. These proteins are coupled to adenylyl cyclase and inward rectifying K^+ channels[47]. They eventually inhibit the influx of calcium into the pre-synaptic neuron. This decreases the fusion of vesicles into the cell membrane, decreasing the release of neurotransmitters[48].

The pre-synaptic receptors were discovered in the 1990s[49]. They are located in neurons and fibers of the myenteric and submucosal plexus of the enteric nervous system,

as well as in the dorsal root ganglia, the dorsal horn of the spinal cord, the brainstem, and vagal efferent neurons[50]. They are usually distributed close to Preyer's patches and submucosal blood vessels[51].

Cannabinoids act on the hindbrain medulla, specifically on the AP, subnuclei of the NTS, and the dorsal motor nucleus of the vagus. In the NTS, they can have effects on the central terminals of primary afferents of the vagus or on interneurons that have synapses with motor neurons[4]. As noted earlier, the NTS is an integrative center in the hindbrain that receives information regarding digestive, cardiovascular, and respiratory information via afferents from the vagus nerve[52]. Cannabinoids can also have an effect on perception from an organ standpoint via peripheral terminals of vagal afferents. It is unclear if endocannabinoids are produced constitutively in the gut, or if they are only produced in response to physiological cues[4].

AEA is one of the endocannabinoids that bind to the CB family of receptors. It was the first one that was reported[4]. It can be produced by the condensation of ethanolamine with arachidonic acid by fatty acid amide hydrolase (FAAH). Also, transacylation by N-acyltransferase forms N-arachidonoyl phosphatidylethanolamine, which is followed by phospholipase D-catalyzed release of AEA[4]. The latter mechanism for production of AEA is more physiologically relevant due to the high K_m of FAAH for ethanolamide. An influx of calcium into post-synaptic cells seems to be the trigger for synthesis[49]. The concentration of this molecule seems to increase after fasting[46]. It enhances basal acetylcholine secretion through a mechanism that does not involve CB receptors, but could involve vanilloid receptors (VRs) on primary afferents. AEA is also responsible for neurokinin receptor internalization in myenteric neurons[4]. It activates

VRs, which can cause intraluminal secretion of substance P in the myenteric plexus, which later leads to inflammation[4]. AEA decreases contractions that are caused by stimulation through the secretion of acetylcholine. It also decreases non-adrenergic, non-cholinergic (NANC) responses by acting presynaptically rather than directly. It does not have any effects post-synaptically on smooth muscle contractility. AEA appears to be responsible for many of the physiological effects of endocannabinoids, including inhibiting upper GI motility[51].

Other endocannabinoids include 2-arachidonoyl-glycerol (2-AG), which is a monoglyceride that is found mostly in brain tissue. This molecule may act more strongly on CB_2 receptors. Noladin ether, or 2-arachidony glyceryl ether is another endocannabinoid[4]. Concentration of 2-AG is increased by an elevation in intracellular calcium, activation of N-methyl-D-aspartate (NMDA) receptors, and in response to LPS. NMDA receptors are found in places that have many CB_1 receptors, for example, the basal ganglia and hippocampus. NMDA receptors are responsive to glutamate, and represent cationic channels that aid in excitatory transmission. They are involved in movement and memory. Cannabinoids influence NMDA receptors to inhibit calcium influx[47].

The CB_1 receptor is responsible for most cannabinoid effects in the central nervous system. The CB_1 receptor is located in the parts of the brain that control impairments in cognition, memory, learning, and motor coordination, including the hippocampus, basal ganglia, cerebral cortex, amygdala, and cerebellum. In the peripheral nervous system, they are located on nerves that innervate the spleen, tonsils, small intestine, urinary bladder, vas deferens, sympathetic nerve terminals, smooth muscle,

adrenal gland, heart, prostate, uterus, and the ovary[47]. They are specifically located on enteric cholinergic neurons[49].

The receptor itself is a G-protein coupled receptor related to the rhodopsin family. Activation of the G protein occurs through three cytosolic loops and a fourth loop that is formed by palmitoylation at the C-terminus of the protein. Agonists interact with the receptor by binding inside of the pore that is formed from this group of helices. Cannabinoids may affect both inhibitory and stimulatory G-proteins, but they mostly have an effect on inhibitory G-proteins that inhibit adenylyl cyclase. This inhibition decreases cAMP concentration within the cell, which decreases activation of PKA. This can have further effects on gene expression and ion influx into the cell through ion channels, including Ca^+ and K^+ channels. Cannabinoids cause a decrease in Ca^+ influx through this mechanism, and this may be how cannabinoids decrease neurotransmitter secretion at presynaptic sites[47].

The CB_1 receptor is produced in dorsal root ganglia neurons, and it is inserted into the membrane of the terminals in the periphery[47]. The receptors are associated with cholinergic neurons within the submucosal and myenteric plexuses and other excitatory nerve fibers[46]. It is implicated in the regulation of water and electrolyte transport in the small intestine. It also plays a protective role regarding inflammatory responses. CB receptor agonists decrease intestinal movement more during an inflammatory state compared to a healthy control state. They increase the threshold for peristalsis while inhibiting the propulsive actions. In the mouse colon, they are also responsible for causing inhibition of cholinergic transmission[4].

The CB_2 receptor also has seven membrane-spanning domains. The receptor works in association with a G-protein. They are mainly located in the periphery of the body, including the spleen, tonsils, and immune cells. They work through acting on adenylyl cyclase and MAP kinases using inhibitory G proteins. They do not, however, effect ion channel function or work with stimulatory G-proteins[47].

The VR is a protein that is activated by noxious stimuli, such as heat, pH, and capsaicin[47]. Capsaicin is known to destroy thin, unmyelinated primary afferents of the dorsal root ganglion (DRG) and nodose making them non-functional[1]. The receptor is typically found on sensory nerves. When the receptor channel is opened, there is an immediate influx of calcium ions. This leads to the release of a neurotransmitter. AEA can dose-dependently activate this receptor by binding to the side that faces the cytoplasm[47].

There is increasing evidence for a receptor for cannabinoids that is not the CB_1 or the CB_2 receptor. WIN 55212-2 is an agonist that inhibits neurotransmission via decreasing pre-synaptic calcium concentrations and by affecting the activation of potassium channels involving G-proteins. It inhibits neurotransmission in the NTS through a receptor that is distinct from those currently known. This has been experimentally shown by Accorsi-Mendonca (2008) because known CB_1 and CB_2 antagonists do not block the effect of WIN on post-synaptic currents. It is also not the same as the $TRPV_1$ receptor due to lack of antagonistic effects. These results show that either there is a different receptor, or that WIN acts via more than one pathway separate from the cannabinoid receptor pathways[52]. Zygmunt (2002) has also shown that antinociception can be induced via a pathway that is independent from CB_1 receptors.

Their experiments showed that the effect of cannabinoids on nerve fibers that are sensitive to capsaicin is not mediated by CB receptors due to lack of antagonistic effects by known CB antagonists. They used VR-1 knock-out mice to confirm that the effects shown are not due to interaction with VRs. This leads to the possibility that there is a new receptor or ion channel that is independent of known cannabinoid receptors and ion channels which may belong to the TRP ion channel family. This unknown receptor may be a helpful target for analgesics and anti-inflammatory drugs, since activation does not cause the usually unwanted psychotropic effects[53].

Endocannabinoids are broken down by reuptake into the cells, movement into the appropriate cellular compartment, and FAAH-mediated hydrolysis. When they are broken down, they yield arachidonic acid and either ethanolamine or glycerol. They can also be broken down by fatty acid oxygenases[4]. Another mechanism by which they may be broken down involves COX-2. Metabolism through this enzyme may produce prostaglandin ethanolamides and glycerol esters. In irritable bowel disease (IBD), the concentration of this enzyme is increased, as are endocannabinoids and CB_2 expression. This may provide other ways for the production of prostamides and may be the cause for changes in the immune system, motility, and secretion in IBD[49]. AEA can also be broken down by anandamide amidohydrolase. This activity can increase inflammation in the gastrointestinal tract[51]. 2-AG is hydrolyzed and added to phospholipids and monacylglycerols in astrocytoma cells, and it is metabolized by monoacylglycerol lipase[4].

Endocannabinoids respond to changes in energy balance and food intake at four different levels within the body, including the limbic system, the hypothalamus and

midbrain, the intestinal system, and adipose tissue[25]. These substances have an effect on the secretion and motility within the gastrointestinal tract as well as antinociceptive and antihyperalgesic properties. Cannabinoids tend to decrease the rate at which gastric contents are emptied and they slow transit through the small intestine[4].

The concentration of endocannabinoids in the hypothalamus is increased when there is a defect in leptin signaling. Neurons that express CB_1 receptors also seem to express CART, specifically in the lateral hypothalamus, retrochiasmatic area, and in the hindbrain[54]. The action of cannabinoids tends to increase the appetite for highly palatable foods, which has both been proven historically and experimentally. Miller[3] performed experiments involving intercerebroventricular injections of CP 55,940 into the fourth and lateral ventricles. These experiments provided evidence for the effect of cannabinoids on the intake of highly palatable foods. It also showed that the region around the fourth ventricle seemed to be more sensitive for orexigenic actions of cannabinoids. However, more recently it has been discovered that low doses and high doses of a cannabinoid agonist have different physiological effects. Low doses tend to increase motor function, while high doses have the opposite effect. The hindbrain has been shown to be a significant player in the control of satiation and ingestion. The dorsal vagal complex gets primary afferent information from oral and gastrointestinal receptors[3].

The functions of cannabinoids in the gastrointestinal tract are fairly well characterized. They slow motility through action on cholinergic contractile responses. They can also inhibit longitudinal muscle reflex contraction, compliance, and maximal ejection pressure. Threshold pressure and volume that are needed to evoke peristalsis are increased. It has been shown that constitutive exposure to cannabinoids can cause

tolerance evoked by downregulation of CB_1 receptor expression at the level of the myenteric plexus[49].

Cannabinoids act through the dorsal vagal complex and dorsal motor nucleus to play an anti-emetic role in pathophysiology. Under illness conditions, diarrhea is decreased in response to the antisecretory role. It may even decrease the growth of colorectal cancer[55]. Cannabinoids are also involved in reward circuits, such as those involving opioids and dopamine[26]. CB_2 receptors are distributed on B cells, NK cells, and mast cells to suppress cell-mediated immunity while increasing adaptive immunity. They also suppress activated macrophages and mast cells[49]. Activation of the CB_1 receptor causes a decrease in gastric acid secretion. This occurs through vagal efferent control of the secretion of parietal cells. It may also decrease the release of histamine[4].

Rimonibant is an antagonist of the CB receptors. It blocks the suppression of excitation of glutamatergic neurons, which causes the release of glutamate. CB receptor agonists generally decrease electrical field stimulation by acting presynaptically to reduce excitatory transmission[47]. WIN55,212-2 is an agonist that inhibits neurotransmitter release from GABAergic terminals. WIN is responsible for a dual response. The first one is a fast response via vagal afferents after an interperitoneal injection or due to direct activation of the receptors. The second response may be due to activation of synergistic circuits[54]. WIN55,212-2 has been shown to decrease lower esophageal sphincter relaxations that are caused by the distention of the stomach of dogs. This action occurs through CB_1 receptor activation through changing peripheral and central vagal pathways[4]. CB_1 receptor antagonists, such as SR-141716A, increase contraction due to electrical stimulation involving mechanisms that occur presynaptically. They increase

the ejection pressure through the gastrointestinal tract. They also increase gastrointestinal motility and defecation. These mechanisms occur via muscarinic receptors[51].

Section VI: The Question

The question that this project will focus on is the effect that a cannabinoid receptor agonist has on vagal afferent activation using CCK as well as their effect on vagal afferent neuron activation and NF-κB and SOCS-3 expression induced by LPS. An antibody to c-Fos is used to detect activation of nodose ganglia neurons in primary culture during this experiment, because there are a number of advantages to using c-Fos to measure neuronal activation. First of all, c-Fos is an easily identifiable and sensitive marker that can be used to identify activated cells *in vivo* after being pharmacologically stimulated. Double staining can also be accomplished when using c-Fos to determine colocalization[56]. It takes approximately one hour to initiate immediate-early gene activation and protein synthesis. Also, the pattern of c-Fos expression allows activation of nodose ganglia neurons to be quantified by counting individual activated cells. Lastly, there is a large database of literature that refers to the use of c-Fos as measurement of neuronal activation that can be used for comparison. The c-Fos protein contains a leucine zipper motif which aids in dimerization with other proto-oncogenes such as the Jun family. The dimers bind at the AP-1 binding domain on DNA, thus it is localized in the nucleus of cells. Since c-Fos is located within the nucleus, it allows for counter-staining with other antibodies that are located elsewhere. C-fos alters gene transcription in response to signals on the cell's surface [57]. C-fos expression is turned on through many different intracellular signaling pathways, including increases in calcium[56].

CCK and Cannabinoids

Vagal afferent fibers express the CB_1 receptor, and its expression is effected by CCK. The concentration of receptors in the membrane increases when an individual is

fasting, and it decreases when CCK is in circulation following feeding[43]. In fact, CCK may have its effect through actions in concert with CB_1. After an animal that has been fasted is refed, CB_1 transcripts are significantly reduced. This may be due to inhibition of transcription or degradation of transcripts. These effects have been demonstrated by an experiment performed by Burdyga *et al* (2004). The increased expression due to fasting may increase signaling through OX-R1 to increase appetite by counter balancing satiety signals[58]. It is likely that cannabinoid agonists have an indirect effect on N-type Ca^{2+} channels to yield a decrease in ACh release from cholinergic nerve fibers[51]. ACh receptors are ligand-gated ion channels that are involved in fast synaptic transmission in both the central and peripheral nervous system[47]. Through experimentation, it has been shown that CCK administration downregulates the expression of CB_1 receptors in the nodose ganglia in the same way that feeding has an effect on CB_1 receptor expression[43]. The mechanism involved with the effect of CCK on CB_1 receptor expression has yet to be determined. This project is looking to see if a CB receptor agonist has the same down-regulating effect on neuronal activation using CCK to further describe the complex interactions involved with feeding behavior.

Inflammation and Vagal Activation Leads to Sickness Behaviors

The next aspect of this project will address the anti-inflammatory effects of cannabinoids. Inflammation causes an acute-phase response that corresponds with fever, increased sleep, and what are known as "sickness behaviors". Symptoms that are included in sickness behavior are weakness, malaise, listlessness, inability to concentrate, depression, lethargy, little interest in surroundings, anorexia, and lack of drinking[59]. This

behavior is considered an important strategy that aids in survival when an individual is infected with a pathogen[60]. The reduced food intake that is associated with sickness and inflammation can cause a reduction in fat reserves and lean muscle mass, which in turn poses a significant health risk in unhealthy individuals[5]. In rat models, LPS administration causes an increase in cytokines such as TNF-α and IL-1β as a good model for inflammation[5].

LPS causes inflammation by first binding to LPS binding protein (LBP) while circulating in the blood stream. LBP transfers LPS to a protein complex that is made up of CD14, a GPI-anchored protein, the accessory protein MD-2, and heterodimerized TLR-4[61]. After binding to TLR-4, LPS activates MyD88, which recruits IL-1 receptor-associated kinase (IRAK) and TNF receptor associated factor (TRAF6). There have been studies conducted by Barbier de la Serre *et al* (2010) that indicate that there is an increase in TLR-4 activation in both ileal epithelium and vagal afferent neurons when these areas are under inflammatory stress due to an increase in LPS as shown through immunoreactivity of the TLR4/MD2 complex[62]. This causes the production of pro-inflammatory transcription factors such as NF-κB[63]. Before NF-κB is activated and translocated into the nucleus, it is bound to the inhibitor protein IκB within the cytoplasm. Activation of another molecule, IKKβ, causes IκB to become phosphorylated and eventually degraded, releasing NF-κB for translocation to the nucleus[64]. This nuclear translocation of NF-κB leads to the transcription of its target genes and the expression of pro-inflammatory cytokines[65] such as TNF-α[66], IL-1β, and IL-6[63]. These cytokines are responsible for causing the physiological symptoms associated with inflammation, including heat, swelling, redness, pain, and loss of function[63]. A negative feedback loop

is also in place that involves expression of suppressor of cytokine signaling-3 (SOCS-3) following the release of pro-inflammatory cytokines, specifically IL-6. Its anti-inflammatory function occurs by inhibiting the association between TRAF6 and TAK1, therefore causing inhibition of MAPK and NF-κB expression induced by LPS[67]. Since SOCS-3 is produced in response to the production of pro-inflammatory cytokines, there should be a decrease in SOCS-3 expression when there is a decrease in the production of cytokines.

Cytokines are the main cause of inflammation during infection. They are involved in the innate immune system, which is responsible for the rapid, non-specific physiological response to foreign substances. They are low-molecular-weight proteins that bind to receptors to induce immune cell differentiation, proliferation, and activity. They are synthesized by macrophages and released during the inflammatory cascade[63]. These peptides do not seem to cross the blood-brain barrier; however there is a transport mechanism that may exist for several cytokines in the central nervous system. The concentration of cytokines that have been shown to enter the brain may not be large enough to activate central mechanisms. Instead, vagal afferent fibers are used to send systemic inflammatory information to the brain[68].

There are two proposed pathways by which the brain monitors peripheral innate immune responses. One of these involves vagal afferent fibers during a state of abdominal and visceral infection. The second pathway involves TLRs in circumventricular organs, such as the area postrema, and the choroid plexus which bind to pathogen-associated molecular patterns (PAMPs), such as LPS. Each of these pathways eventually cause microglial cells to produce pro-inflammatory cytokines within

the brain itself[60]. TNF- α and IL-1 are examples of pro-inflammatory cytokines that generally have an effect on sites that are close to where they are released. Increased levels of these specific cytokines are correlated to the severity of the illness and may be considered mediators of sepsis[63].

Cytokines travel to the central nervous system through the bloodstream. They communicate with the brain through a variety of mechanisms including transport, direct entry at circumventricular organs, and binding to receptors on endothelial cells[69]. The neural pathway involves cytokines that signal the CNS by activating vagal afferent neurons through paracrine action at the site of production. Experiments have shown through subdiaphragmatic vagotomy that an intact vagus nerve is necessary for LPS and cytokines to have their normal physiological effect[69]. It is possible that the cytokines activate vagal afferent fibers through binding to receptors on paraganglia that then synapse with vagal afferent fibers that communicate with the NTS[69]. Those fibers then communicate with the DMN. The efferent portion of this inflammatory reflex has been termed the "cholinergic anti-inflammatory pathway," because ACh is used to suppress TNF synthesis and inhibits the release of IL-1, IL-6, and IL-8[63]. These efferent fibers send signals back to the gastrointestinal tract. ACh decreases cytokine release through the α7 nicotinic acetylcholine receptor, which is located on residential macrophages and other cells, such as dendritic cells and mast cells[70]. Experimentally, it has been shown that central inhibition of IL-1β prevents many of the acute phase illness responses from occurring[68]. In addition, vagotomy blocks the induction of the production of IL-1β mRNA in the brain of LPS-treated mice. This indicates that IL-1β may be an important part of the acute phase response that is induced centrally via vagal-mediated signals[68].

Cannabinoids have an effect on the innate immune system and inflammation through five different pathways. They inhibit cell proliferation, cytokine and chemokine production, and bone marrow-derived myeloid cell recruitment while inducing regulatory T-cell recruitment and apoptosis. These actions support the potential for use of cannabinoids in treatment for inflammatory diseases. Cannabinoids decrease hypothalamus-pituitary-adrenal (HPA) axis activity and help with adaptation of the HPA axis and the response to stress while decreasing anxiety behaviors. They are also involved with analgesic responses to stress situations. Cannabinoids in the brain may be involved with neurogenesis and have some neuroprotective properties. They have also been found to have epithelial wound healing properties in cases of inflammatory bowel disease[71]. Cannabinoids have been shown to induce wound-closure in human colonic epithelial cell lines. This indicates that delayed wound healing, which is associated with IBD lesions, could be inhibited by cannabinoids[72].

AEA, an endocannabinoid, acts as an anti-inflammatory agent. This has been shown through the use of an antagonist to increase inflammation and an agonist to decrease it in two different models of gastrointestinal inflammation, one using dextran sulphate sodium and another using intrarectal infusion of dinitrobenzene sulfonic acid. When the amount of gastrointestinal inflammation was assessed in a knock-out model for the CB_1 receptor, it was shown that there were higher levels of inflammation in "knock-outs" compared to control animals[73]. There are more CB_1 receptors in the inflamed gut in a model induced using croton oil, indicating that there is an up-regulation during inflammation. This implies that they are more active in inhibiting contraction during an inflammatory state. Also, mice are less susceptible to gastrointestinal inflammation when

they are deficient in FAAH[55]. There is also a quicker endocannabinoid turnover rate in the inflamed gut[51].

Inflammation and Gut Motility

In addition to sickness behaviors previously described, inflammation also has an effect on gut motility. Normal gastrointestinal motility is controlled by three different key systems of the body, including the parasympathetic and sympathetic nervous systems, the enteric nervous system and the glial cells associated with it, and the intestinal contractile unit that involves interstitial cells of Cajal and smooth muscle[74]. There are two different reflexes involved with the parasympathetic and enteric nervous systems. The descending reflex transmits a signal that originates from the intestinal tract as the intestinal wall is distended due to increased volume. This signal travels through interneurons to inhibitory motor neurons where ATP and NO are released to cause relaxation of the area anal to the bolus. The ascending reflex involves transmission through interneurons to excitatory motor neurons that use ACh and tachykinins to contract the portion of the intestinal wall that is oral to the bolus moving through it. These two processes work together to move the bolus through the gastrointestinal tract towards the anal end[17].

There are many different mediators that contribute to the pathogenesis of gut inflammation, including nitric oxide, prostaglandins, and cytokines. These molecules could be involved in motility, sensitivity, and pain disturbances[70]. Monocytes are also involved, and they secrete monocyte-chemoattractant protein-1, which recruits more

monocytes to the site of infection, which causes an increase in production of cytokines and other molecules that have an effect on gut motility[70].

There are three major areas where cannabinoids can affect the process of motility[17]. CB_1 receptors in the myenteric plexus reduce cholinergic neurotransmission. Agonists bind to receptors on pre-synaptic neurons to inhibit contraction in different parts of the gastrointestinal tract by decreasing the release of ACh[55]. This causes a decrease in gastrointestinal motility *in vivo*. For example, the rate of colonic propulsion and defecation has been assessed by measuring the amount of time it takes for a mouse to expel a glass bead. In this model, AEA decreased the rate of propulsion. This endocannabinoid effect has also been shown by using rimonibant, an antagonist, to increase motility in a guinea pig model and by using an uptake inhibitor to decrease gastric propulsion in mice[73]. The mechanism that cannabinoids work through in order to decrease motility involves reducing acetylcholine secretion from prejunctional neurons. It is also possible that inhibition of NANC neurons is involved[55].

Inflammatory bowel disease is a severe problem in the United States, effecting over a million people. The syndrome is described by abdominal pain, discomfort, and irregular bowel movements for at least three days per month over three months. Gastrointestinal motility is usually increased during this syndrome[75]. Possible treatments for gastrointestinal inflammation include FAAH inhibitors. These substances do not cause the high feeling that other cannabinoid agonists evoke, but they still have the anti-inflammatory effect. They work by decreasing leukocyte infiltration due to decreasing the release of chemokines and cytokines[76]. Secretion of TNF-α is also decreased[49].

Section VII: Conclusion

The vagus nerve innervates many different organs, and this innervation is integral in normal, everyday function involving the respiratory, cardiovascular, and gastrointestinal systems. There are afferent, efferent, and central pathways that communicate with each other to sense stimuli and to cause appropriate reactions to those stimuli. Many different neuropeptides are used within the gastrointestinal system, usually involving the vagus nerve, to control satiation and appetite. Cannabinoids play an important role in many aspects of gastrointestinal function, appetite, and during infection and inflammation. For example, they can have anti-inflammatory effects, reduce sickness behavior, and reduce gastrointestinal motility. There are therapeutic possibilities for cannabinoids in states of illness that result in gastrointestinal dysfunction, such as inflammatory bowel disease. They can also help to increase appetite and decrease sickness behaviors in the elderly and those undergoing chemotherapy. These possibilities are important not only within the human population but also in the pet population, and it is of vital importance to find a way to relieve the symptoms of these disease states.

Section VIII: Experimental Design

This project is divided into three aims:

AIM 1 studies will determine the magnitude of activation of vagal afferent neurons by CCK at various concentrations. Additionally, a CB receptor agonist will be

used to determine the effects that cannabinoids have on activation of vagal afferent neurons with CCK. Lastly, the CB_1 inverse agonist, AM251, will be used to confirm the action of CB agonists on activation with CCK (Figure 1-1).

AIM 2 studies will determine the magnitude of activation of vagal afferent neurons by LPS at various concentrations. A CB receptor agonist will be used once again to determine the effect on activation of vagal afferents with LPS. AM251 will also be used to confirm action of CB agonists on activation with LPS through the CB_1 receptor (Figure 1-2).

AIM 3 studies will show the effect that LPS has on NF-κB, pNF-κB, and SOCS-3 expression in vagal afferent neurons to give insight into the molecular effects and signal transduction pathways invoked through treatment with LPS and cannabinoids.

The Hypotheses

Hypothesis 1: CCK will activate vagal afferent neurons harvested from nodose ganglia in a concentration dependent manner, and CP 55,940, a CB receptor agonist, will decrease the activation observed *in vitro*.

Hypothesis 2: LPS will activate vagal afferent neurons in a concentration dependent manner, and CP 55,940 will attenuate that activation *in vitro*.

Hypothesis 3: LPS will increase the protein expression of SOCS-3 in nodose ganglia tissue, and LPS will decrease the amount of pNF-κB and NF-κB in whole nodose ganglia *in vitro* as shown by Western blot. CP 55,940 will attenuate the effects seen on NF-κB, pNF-κB, and SOCS-3 expression after treatment with LPS.

The Objectives

Objective 1: To show for the first time that cannabinoids decrease activation caused by CCK in vagal afferent neurons *in vitro*.

Objective 2: To show for the first time that cannabinoids decrease activation caused by LPS in vagal afferent neurons *in vitro*.

Objective 3: To show that treatment with LPS has an effect on the expression of NF-κB, pNF-κB, and SOCS-3 in whole ganglia and to show for the first time that cannabinoids have an attenuating effect on the expression of NF-κB, pNF-κB, and SOCS-3 in whole nodose ganglia after treatment with LPS *in vitro*.

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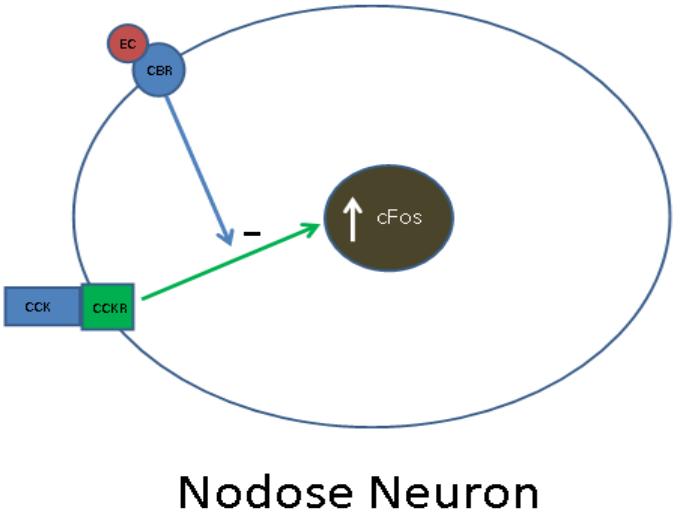

Figure 1-1. This is a visual representation of the hypothesis for the first objective, which is that CP 55,940, a CB receptor agonist, will attenuate activation of nodose ganglion neurons by CCK through indirect downstream actions by binding to the CB_1 receptor.

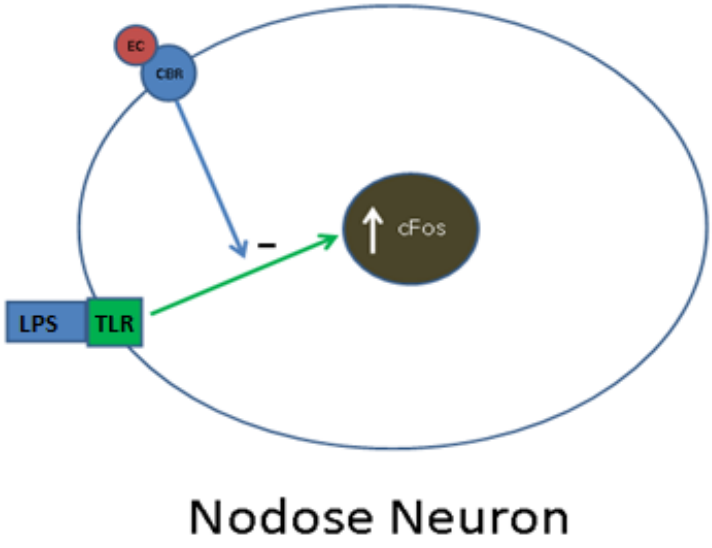

Figure 1-2. This is a visual representation of the hypothesis for the second objective, which is that cannabinoids will attenuate activation of nodose ganglion neurons induced by LPS indirectly through downstream effects. When LPS is released, there is an increase in activation as shown by an increase in cFos within the nucleus. Our hypothesis is that cannabinoid binding to CB_1 will decrease the ability for LPS to activate vagal afferent neurons.

CHAPTER 2

ACTIVITY IN NODOSE GANGLIA NEURONS AFTER TREATMENT WITH CP 55,940 AND CHOLECYSTOKININ[1]

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Abstract

Previous work has shown that cannabinoids increase feeding, while cholecystokinin (CCK) has an anorexigenic effect on food intake. Receptors for these hormones are located on cell bodies of vagal afferent neurons in the nodose ganglia. An interaction between CCK and cannabinoid receptors has been suggested[1]. The purpose of these studies is to explore the effect of pretreatment with a cannabinoid agonist, CP 55,940, on nodose neuron activation by CCK. To determine the effect of CP 55,940 and CCK on neuron activation, rats were anesthetized and nodose ganglia were extirpated. The neurons were dissociated and plated. The cells were treated with media, CP 55,940, CCK, CP 55,940 followed by CCK, or AM 251, a CB_1 receptor inverse agonist, and CP 55,940 followed by CCK. Immunohistochemistry was performed to detect nuclear cFos as a measure of cell activation. Neurons were identified using neurofilament immunoreactivity. Total neurons on each slip were counted using fluorescence imaging, and the number of neurons that were cFos positive was counted in order to calculate the percentage of activated neurons per coverslip. Pretreatment with CP 55,940 decreased the percentage of neurons expressing cFos-immunreactivity in response to CCK. This observation suggests that cannabinoids inhibit CCK activation of nodose ganglion neurons.

Introduction

The vagus nerve has a variety of effects on thoracic and abdominal function[2]. For example, vagal afferent fibers respond to gastric distention due to presence of food in the gut through pressure sensors and chemical stimuli[3]. This neural input aids in the control of digestion and food intake. The vagus nerve slows gastric emptying and is involved in esophageal, stomach, and duodenal contractions through the vago-vagal reflex. This reflex involves afferent fibers with cell bodies located within the nodose ganglia that send information to the nucleus of the solitary tract (NTS), then interneurons project to the dorsal motor nucleus of the vagus (DMN), and lastly efferent fibers exit the brain to either stimulate or inhibit smooth muscle of the esophagus, stomach, and small intestine[4]. There are numerous neuropeptides and neurotransmitters that affect vagal afferent firing to control digestion and food intake. Cholecystokinin (CCK) and cannabinoids are examples of such neurochemicals[4].

CCK is produced in I cells in the duodenal and jejunal mucosa and is released in the brain and the enteric system in response to intake of protein and fatty acids that have more than 12 carbons. The prepropeptide is processed by endoproteolytic cleavage to create the active peptide CCK-8. This peptide binds to two different receptors, CCK-1 and CCK-2. CCK-1 receptor (CCK-1R) is located mostly in the gastrointestinal tract, and it is found on the gastric, celiac, and hepatic branches of the vagus nerve that communicate with the stomach, jejunum, and the hindbrain. Administration of CCK increases vagal afferent firing in the hindbrain region by action through binding to the

CCK-1R[3]. The CCK-2R is primarily found in the brain, notably in the hypothalamus and the hippocampus[5].

Endocannabinoids are endogenous lipophilic molecules formed from membrane glycerolphospholipids that are synthesized and secreted to physiological demand[6]. Examples of ligands that bind to cannabinoid receptors include anandamide (AEA) and 2-arachidonoyl glycerol (2-AG), as well as synthetic molecules, such as the agonist CP 55,940[7]. These molecules are often produced in response to depolarization of the post-synaptic neuron and have presynaptic membrane effects[7]. They are reported to be released from many neurons associated with control of the gastrointestinal system. For example, these molecules are released from neurons of the myenteric and submucosal plexuses of the gut, neurons in the dorsal root ganglia, within the dorsal horn of the spinal cord, the brainstem, and from vagal efferent neurons[6]. Within the medulla, abundant receptors are located in the NTS and the area postrema (AP), two areas important to visceral sensation[8]. In the NTS, they are reported to act at central terminals of primary afferent fibers of the vagus nerve or on interneurons that synapse with motor neurons[8].

It has been demonstrated that the CB_1 receptors are synthesized in dorsal root ganglia neurons and are transported and inserted into the membrane of the terminals in the periphery[7]. The receptors are associated with cholinergic neurons within the submucosal and myenteric plexuses of the enteric nervous system and other excitatory nerve fibers[6]. The CB_1 receptor is implicated in the regulation of water and electrolyte transport in the small intestine and may also play a protective role during inflammatory responses[8]. The CB_2 receptors are found on peripheral tissues of the body, including the spleen, tonsils, and immune cells[7].

The small intestine secretes both CCK and endocannabinoids, and they are suggested to act to control food intake[9]. CCK is released in response to food entering the gastrointestinal tract and activating vagal afferent fibers with a subsequent decrease in food intake and delayed gastric emptying[9]. This may help match the amount of diet delivered to the duodenum to the amount that is capable of digestion by stimulating secretions from the pancreas and contraction of the gall bladder, so that enzymes and bile salts are appropriately delivered[4]. AEA and 2-AG are secreted by neuronal activation and increase food intake while also slowing gastric emptying by reducing acetylcholine secretion[8]. In summary, cholecystokinin and endocannabinoids have antagonistic effects on food intake by action through vagal afferent fibers.

While cannabinoid effects on gastrointestinal motility may be non-vagal, a study conducted by Burdyga *et al* showed that CB_1 receptors are located on neurons of the nodose ganglia. When rats were fed ad libitum, they found CB_1 receptor expression located in the rostral part of the ganglion. When rats were fasted for 48 hours, they reported an increase in immunoreactivity in the mid and caudal regions of the ganglion. Many of these vagal afferent neurons, specifically those that originate from the stomach and duodenum, are located around the periphery of the ganglion and contain both CB_1 and CCK-1 receptors. This research group also reported that after re-feeding following fasting, there is a decrease in CB_1 mRNA expression compared to what was observed during the fasted state. A decrease in CB_1 receptor mRNA concentration similar to the decrease observed following re-feeding was observed when fasted rats were injected with CCK-8. Therefore, CCK-8 administration seems to have an effect on mRNA expression of CB_1 receptors[1]. Based on these observations we hypothesized that treatment with CP

55,940, a cannabinoid receptor agonist, will decrease activation of nodose ganglion neurons following treatment with CCK.

Materials and Methods

Adult male Sprague-Dawley rats (200-350g) from Harlan Laboratories, Indianapolis, IN were used in all studies. The animals were housed in pairs in standard shoebox cages at 22ºC with a 12:12 light:dark cycle and given access to food and water ad libitum. All research using animals was approved by the University of Georgia Institutional Animal Care and Use Committee.

Extraction of Nodose Ganglia

Non-fasted rats were anesthetized with a cocktail of ketamine (50mg/kg of body weight), acepromazine (3.3mg/kg of body weight), and xylazine (3.3mg/kg of body weight). The rat was positioned in dorsal recumbency and an approximately 4cm incision was made in the neck over the trachea. The vagus nerve was identified using blunt dissection techniques to separate the digastric, sternocleidomastoid, and sternothyroid muscles to locate the nerve coursing beside the carotid artery. The nodose ganglia were identified near the posterior lacerated foramen and extracted. Once removed, the nodose ganglia were placed in Hibernate solution (Brain Bits; Springfield, IL) on ice until they were dispersed for cell culture. Following extirpation of the nodose ganglia, the rat was humanely euthanized.

Cell Isolation and Culture

Primary cells were isolated and cultured according to the procedure outlined by Simasko *et al*[10]. Both the left and the right nodose ganglia from an animal were combined for each cell isolation procedure. The ganglia were transferred along with 2-3mL of Hibernate solution into a 35mm culture dish. The ganglia were de-sheathed under a dissecting scope using sterile forceps and a 23-gauge needle. Then, they were transferred into enzyme digestion solution (3mg dispase, 3mg collagenase, 3mL Hank's Balanced Salt Solution without Calcium and Magnesium). The ganglia were minced using a scalpel and forceps. The dish was placed in an incubator (37^{o}C, 5% CO_2) to allow digestion for 90 minutes. Once the dish was removed, approximately half of the solution from the dish was pipetted into a 15mL conical centrifuge tube using a pipette coated with Sigmacote (Sigma-Aldrich; Saint Louis, MO). Then, using the same pipette, the ganglia were triturated in the dish. Following that, the ganglia were transferred into the centrifuge tube along with the rest of the solution in the culture dish. With a plain pipette, the culture dish previously containing enzyme digestion solution was filled with media made up of HEPES-buffered DMEM supplemented with antibiotic (penicillin and streptomycin) and 10% Fetal Bovine Serum as to rinse the dish of any cells left. This media was transferred to the conical tube containing the minced ganglia. This was repeated until there was 10mL of media in the conical tube. Then, the tube was centrifuged at 140G for 1.5 minutes with no brake on. Once the tube was removed from the centrifuge, the supernatant was removed until 1.0mL was left in the bottom. With pipettes coated with Sigmacote, the pellet was re-suspended and more media was added until the total amount in the tube was 10mL. Centrifugation was repeated. Finally, with

another pipette coated with Sigmacote, the supernatant was removed once again until 1.0mL remained at the bottom. The pellet was re-suspended, and the solution was divided between six coverslips that had been pre-treated with 0.1mg/mL of poly-L-lysine hydrobromide (Sigma-Aldrich; Saint Louis, MO). The cells were placed in the incubator for approximately three hours, and then enough media was added to cover the coverslip and the slips were returned to the incubator. The following morning, the old media was removed and new media was added to each of the dishes.

Cell Treatment

The cells were incubated in HDMEM + 10% FBS for 1 week before they were treated with either media (control), 1µM CP 55,940 (Tocris; Bristol, UK), 0.1µM CCK (American Peptide Company; Sunnyvale, CA), 1.0µM CCK, 3µM CCK, 10µM CCK, 1µM CP 55,940 with 1µM CCK, 1µM CP 55,940 with 3µM CCK, or 1µM AM251 (Tocris; Bristol, UK) with 1µM CP 55,940 and 1µM CCK. When treated with just CP 55,940, the CP 55,940 was left on the cells for fifteen minutes, and then it was replaced with media for 90 minutes. The CCK treatments were left on for 90 minutes. The CP 55,940/CCK studies were performed by treating the cells with CP 55,940 for fifteen minutes, and then replacing the CP 55,940 with CCK for 90 minutes. The AM/CP 55,940/CCK studies were conducted by combining the AM251 and CP 55,940 and treating the cells with the combined drugs for fifteen minutes, then replacing the solution with CCK for 90 minutes. After treatment, the cells were fixed by immersion in 4% paraformaldehyde.

Immunohistochemistry

The coverslips were first incubated in 0.3% H_2O_2 for an hour. Then, they were blocked in 4% normal goat serum for two hours. Two different antibodies were used in this experiment. The first antibody was rabbit polyclonal anti-cFos from Calbiochem (Darmstadt, Germany) (1:40,000), and the second antibody was mouse monoclonal anti-neurofilament heavy chain from Sigma-Aldrich (Saint Louis, MO) (1:500). This anti-neurofilament antibody recognizes both the phosphorylated and non-phosphorylated forms of neurofilament within culture. The cells were incubated in these antibodies together for approximately 72 hours. Following incubation, they were washed three times for twenty minutes in phosphate buffered saline (PBS) with Triton. Then, the protocol was followed from the rabbit Vectastain ABC Kit (Vector Laboratories, Inc; Burlingame, CA), and DAB peroxidase substrate (Vector Laboratories, Inc; Burlingame, CA) was used as the chromagen for the cFos staining. After the DAB was developed, the cells were washed three times for twenty minutes in PBS with Triton, and the mouse anti-goat AlexaFluor 488 or 546 from Molecular Probes (Invitrogen; Grand Island, NY) was used as a fluorescent antibody to visualize the neurons in culture. Elimination of primary and secondary antibodies from the protocol was used as controls to ensure staining was specific.

Cell Counting

The total number of neurons stained with fluorescent secondary antibody to neurofilament was counted using a fluorescent Nikon UFX-IIA microscope. Those neurons that were also stained for cFos were counted as well. The researcher counting

the cells was blinded to the treatment group of each coverslip. The percentage of cFos positive neurons was calculated based on the number of neurons that had been stained with cFos divided by the total number of neurons. These numbers were compared between the different treatment groups.

Statistical Analysis

A one-way ANOVA test was used to determine whether the means of the treatment groups are significantly different at a $p < 0.05$ level. When ANOVA indicated significant differences, a Dunnett's test or Newman-Keuls Multiple Comparison test was utilized to determine which specific treatments were significantly different from one another ($p < 0.05$). When appropriate, a two-way ANOVA test was performed to determine whether or not there was a significant interaction between two different variables.

Results

Activation of nodose ganglia neurons with CCK

Examples of cells that were stained with neurofilament or cFos are illustrated in Figure 2-1. On average, there were 176 neurons per coverslip that stained positive for neurofilament as shown by immunofluorescence. Of these neurons, 25% of the control neurons (n = 11 coverslips) on average stained positive for cFos, indicating that these neurons had been activated. Thirty-five percent of the neurons that were treated with 0.1µM CCK (n = 12 coverslips), 47% of them treated with 1.0µM CCK (n = 10

coverslips), 58% treated with 3.0µM CCK (n = 12 coverslips), and 67% treated with 10.0µM CCK (n = 15 coverslips) also stained positive for cFos (Figure 2-2). Single factor ANOVA (df = 4) shows that treatment with CCK has a significant effect on the percent of neurons activated ($p = 8.26 \times 10^{-21}$). The data indicate a clear correlation between the number of activated nodose ganglia neurons and the concentration of CCK that was used to activate them, suggesting a dose dependent effect on vagal afferent neuron activation.

Effect of CP 55,940, a cannabinoid agonist, on neuron activation

Coverslips treated with CP 55,940 (1µM) for fifteen minutes prior to being incubated with media for 90 minutes revealed that there were 43 neurons on average that stained positive for neurofilament on these coverslips (n = 14 coverslips). Of these, 26% also stained positive for cFos (Figure 2-3). This number did not significantly differ from the percentage of neurons that stain positive for cFos under control conditions ($p = 0.33$). Therefore, CP 55,940 by itself did not alter cFos activation compared to control.

Effect of pretreatment of neurons activated by CCK with CP 55,940

Nodose ganglia neurons were treated with CP 55,940 (1µM) for fifteen minutes prior to treatment with either 1µM (n = 11 coverslips) or 3µM CCK (n = 12 coverslips) for 90 minutes. On average, 117 neurons treated with CP 55,940 and 1µM CCK stained positive for neurofilament. Approximately 28% of those neurons treated with 1µM CCK after pretreatment with CP 55,940 showed cFos immunoreactivity compared to 47% without pretreatment with CP 55,940 (Figure 2-3). This demonstrated a significant

decrease in neuron activation after pretreatment by CP 55,940 ($p = 1.10x10^{-7}$). Moreover, this percentage activation was not significantly different from control ($p = 0.12$). Of the 80 neurons on average per coverslip that were pretreated with CP 55,940 and activated with 3μM CCK, 37% stained positive for cFos (Figure 2-4). This was significantly different from the percentage activated with 3μM CCK ($p = 9.49x10^{-9}$). This percentage activated was also significantly different from control ($p = 1.59x10^{-5}$). Two-factor ANOVA (df = 2) shows that treatment with CCK and pre-treatment with CP 55,940 has a significant effect on the percent of neurons activated and also that there is an interaction between those two variables ($p = 5.2x10^{-11}$). These data suggest that cannabinoid action decreases activation of vagal afferent neurons by CCK.

Effect of the cannabinoid reverse agonist, AM251, on the reversal of activation with CCK by cannabinoid agonists

The last treatment group was then treated with a combination of 1μM AM251 and 1μM CP 55,940 for fifteen minutes and then treated with 1μM CCK for 90 minutes (n = 12 coverslips). AM251 has an approximately four fold greater affinity for the CB_1 receptor than CP 55,940[11]. Coverslips that were pretreated with AM251 in conjunction with CP 55,940 revealed that 46% of the 203 neurons on average per coverslip that stained positive for neurofilament also stained positive for cFos (Figure 2-3). This number was significantly different from control ($p = 9.42x10^{-10}$), but it was not significantly different from the percentage of neurons activated with 1μM CCK ($p = 0.39$). When coverslips were treated solely with AM251 as a control (n = 8 coverslips), the average percentage of neurons positive for cFos was 33% of the 125 neurons per

coverslip (Figure 2-3). This was significantly different both from the control ($p = 3.38x10^{-4}$) and from the AM/CP 55,940/CCK treatment ($p = 3.23x10^{-6}$). Two-factor ANOVA (df = 1) shows that treatment with AM/CP 55,940/CCK and CP 55,940/CCK has a significant effect on the percent of neurons activated and that there is an interaction between those two variables ($p = 1.63x10^{-6}$). Therefore, by blocking binding of CP 55,940 to CB_1 receptors with AM251, the inhibition of neuronal activation was abolished. This indicates that the decrease in CCK activation of nodose ganglion neurons by CP 55,940 involves the CB_1 receptor.

Discussion

Our working hypothesis was that cannabinoids acting via the CB_1 receptor decrease activation of nodose ganglion neurons induced by treatment with CCK. Our results indicate that pre-treatment with a CB receptor agonist, CP 55,940, causes a decrease in nodose neuron activation by CCK of neurons in culture. Specifically, our data indicate that treatment of vagal afferent neurons with CCK increases cFos immunoreactivity. This activation correlates with the concentration of CCK applied to the neurons in culture and illustrates the dose dependence of this effect. Once we established the action of CCK to increase cFos immunoreactivity in nodose ganglion neurons, we were able to demonstrate that pretreatment with CP 55,940 decreased neuron activation occurring in response to 1µM and 3µM CCK. It is important to note that the dose of CP 55,940 we used was less effective against activation of neurons by 3µM CCK than through activation by 1µM CCK. This likely reflects the greater stimulation of the

nodose ganglion neurons by the higher concentration of CCK as suggested by our dose-response study illustrated in Figure 2-2. Finally we demonstrated that treatment of the nodose ganglion neurons with AM251 almost completely reversed the effect of CP 55,940. This suggests that the decrease in activation to CCK by CP 55,940 is due to binding to the CB_1 receptor. It is noteworthy that treatment with AM251 alone showed a significant increase in neuronal activation compared to control. This suggests the possibility of constitutive activation of CB_1 receptors in this population of cultured neurons and glial cells as AM251 is an inverse agonist.

CCK is released by intestinal cells in response to food intake as a satiety signal to the brain[9], while endocannabinoid release increases during the fasted state to increase food intake[8]. Receptor expression on vagal afferent neurons changes according to the animal's fed or fasted state. There is an increase in CB_1 receptor expression during the fasted state, while there is a decrease in expression of Y2 receptor (Y2R) and cocaine-and-amphetamine-related transcript (CART). PYY, which binds to the Y2R, is secreted in response to food intake, whereas CART expression is modulated by CCK release during an anorexigenic or fed state. Therefore, during a fed state, there is an increase in CCK as well as an increase in Y2 and CART and a decrease in the expression of the CB_1 receptor. In summary, at least two different vagal afferent phenotypes exist. Vagal afferents can either show the "hunger" phenotype or the "satiety" phenotype[12].

It has been shown that activation of the CCK-1 receptor causes CREB phosphorylation through a PKC-dependent pathway, which increases expression of CART and Y2 receptors[13]. Our results suggest that endogenous cannabinoids could impact the phenotype of vagal afferent neurons. Cuellar and Isokawa have shown that 2-

AG inhibits ghrelin-induced phosphorylation of CREB[14]. Therefore, endocannabinoid binding to the CB_1 receptor has been shown to decrease the amount of CREB phosphorylation in neuronal populations. It is possible that the decrease in neuronal activation by CP 55,940 in response to CCK is due to action involving the CREB pathway by affecting CREB phosphorylation. This phosphorylation can occur in response to calcium influx, and it has been shown that ligand binding to the CB_1 and CCK-1 receptors has an effect on calcium concentration within the neuron (unpublished observation). In addition, CREB regulates c-Fos expression within the cell, which further supports the possibility that our results are due to action through this specific pathway. Future studies will evaluate the ability of CP 55,940 to impact CREB phosphorylation and calcium signaling.

The physiologic role of cannabinoids in the vagal afferent system is still unclear. Cannabinoids have historically been used in the treatment of gastrointestinal pain, flatulence, gastroenteritis, Crohn's disease, diarrhea, and diabetic gastroparesis, and are reported to cause an increase in appetite[6]. Our data suggest that one role of cannabinoids in the visceral afferent system is to antagonize the actions of CCK. This could impact food intake and gastrointestinal motility. The potential for therapeutic benefit from cannabinoids is significant. There are many different instances when increasing appetite is beneficial. The elderly, those undergoing chemotherapy, and the chronically ill all experience a decrease in appetite, which leads to a decrease in energy intake. Energy is required for appropriate immune and inflammatory responses to fight infection and allow regeneration of damaged tissue. Identifying peripheral targets for cannabinoid action is

important as these targets may provide a means of allowing beneficial effects of the cannabinoid while avoiding the central neural effects, such as dysphoria.

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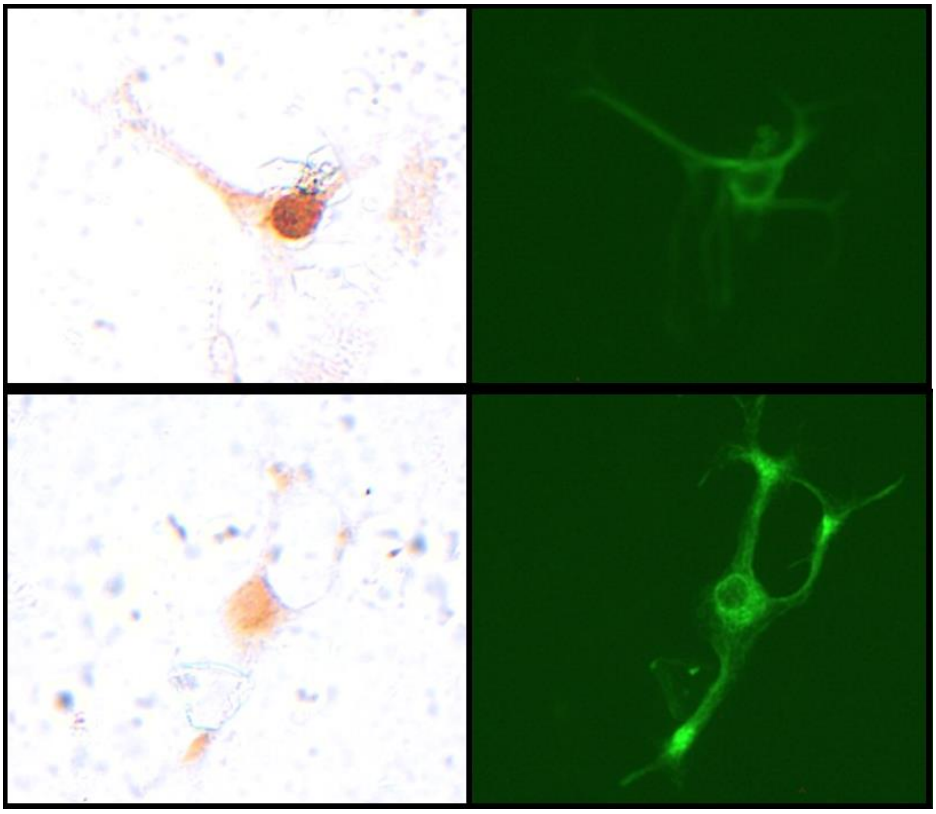

Figure 2-1. Neurons that have stained positive for neurofilament (right panels) and illustrate either positive (top) or negative (bottom) cFos immunoreactivity (left panels).

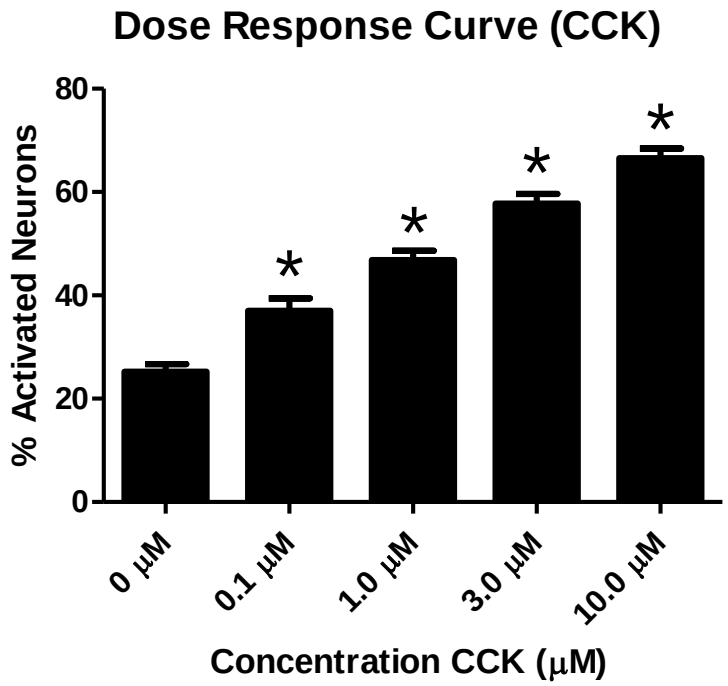

Figure 2-2. Average percentage of neurons that are activated by media, 0.1μM CCK, 1.0μM CCK, 3.0μM CCK, and 10μM of CCK per coverslip (n = 11, 12, 10, 12, and 12 coverslips respectively). 25% of neurons are activated under control conditions. 0.1μM CCK activates 35% of neurons on average from each coverslip, while 1.0μM CCK activates 47% of neurons. 3μM CCK activates 58%, and 10μM CCK activates 67% of neurons. *Significantly different from control ($p < 0.05$)

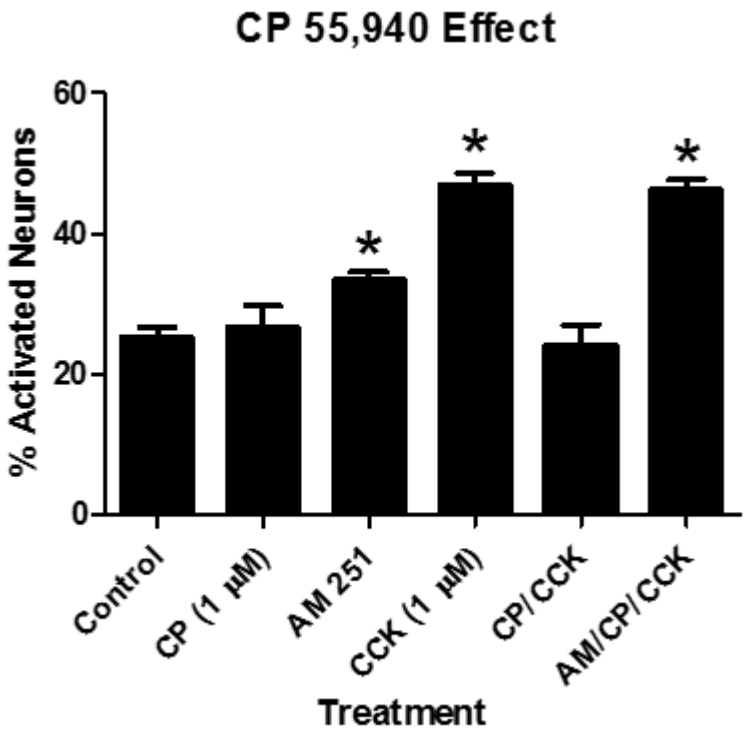

Figure 2-3. Percentage of neurons that are activated with media, 1µM CP 55,940, 1µM AM251, 1µM CCK, 1µM CP 55,940/1µM CCK, and 1µM AM251/CP 55,940/CCK (n = 11, 14, 8, 10, 11, and 12 coverslips respectively). Control = 25%, CP 55,940 (1µM) = 26%, AM251 (1µM) = 33%, CCK (1µM) = 47%, CP 55,940/CCK = 28%, AM/CP 55,940/CCK = 46%. *Significantly different from control ($p < 0.05$)

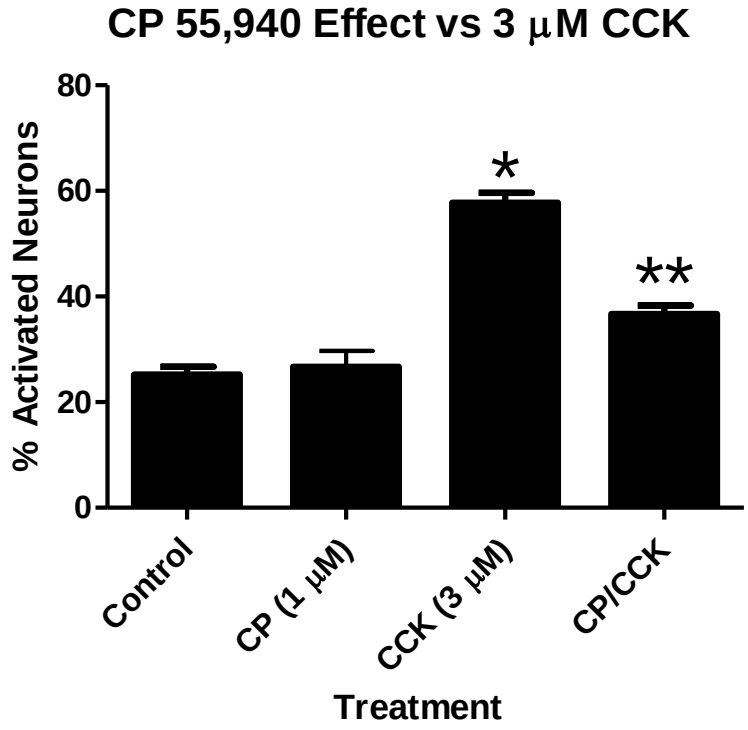

Figure 2-4. Percentage of neurons that are activated with 3μM CCK and pretreated with CP 55,940. Control = 25%, CP 55,940 (1μM) = 26%, CCK (3μM) = 58%, CP 55,940/3μM CCK = 37% (n = 11, 14, 12, and 12 coverslips respectively). *Significantly different from control ($p < 0.05$) **Significantly different from 3μM CCK ($p < 0.05$)

CHAPTER 3

ACTIVITY IN NODOSE GANGLIA NEURONS AFTER TREATMENT WITH CP 55,940 AND LIPOPOLYSACCHARIDE[1]

[1]Johnston, JR, Freeman KG, and Edwards GL.
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Abstract

Previous work has shown that cannabinoids have an anti-inflammatory action during challenge with lipopolysaccharide (LPS). Cannabinoid receptors are located on cell bodies of vagal afferent neurons in the nodose ganglia. An interaction between signaling pathways involving LPS and cannabinoids has been suggested[1]. The purpose of these studies is to explore the effect of pretreatment with a cannabinoid agonist, CP 55,940, on nodose neuron activation by LPS. To determine the effect of CP 55,940 and LPS on neuron activation, rats were anesthetized and nodose ganglia were extirpated. The neurons were dissociated and plated. The cells were treated with media, CP 55,940, LPS, CP 55,940 followed by LPS, or AM251, a CB_1 receptor inverse agonist, and CP 55,940 followed by LPS. Immunohistochemistry was performed to label the cells for cFos as a measure of cell activation. Neurons were identified using neurofilament immunoreactivity. The neurons on each slip were counted using fluorescence imaging, and the number of neurons that were cFos positive was counted in order to calculate the percentage of activated neurons per coverslip. Pretreatment with CP 55,940 decreased the percentage of neurons expressing cFos-immunreactivity in response to LPS. This observation suggests that cannabinoids inhibit LPS activation of nodose ganglion neurons.

Introduction

Gram negative bacteria have lipopolysaccharide (LPS) located on their surface. LPS is a large molecule that is made up of lipid and polysaccharides that causes systemic inflammation through the production of pro-inflammatory cytokines[2]. These cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6, are produced in response to a host cell's interaction with LPS on the bacterial surface through binding to toll-like receptor-4 (TLR-4). Pro-inflammatory cytokines affect many systems in an organism, and they are responsible for causing the symptoms associated with inflammation, including heat, swelling, redness, pain, and loss of function[3].

LPS causes a systemic response through the interaction of many different proteins. LPS binding protein (LBP) circulates through the bloodstream and binds to and transfers LPS to a protein complex that is made up of CD14, a GPI-anchored protein, the accessory protein MD-2, and heterodimerized TLR-4[4]. After binding to TLR-4, LPS activates MyD88, which recruits IL-1 receptor-associated kinase (IRAK) and TNF receptor associated factor-6 (TRAF6), which causes production and activation of pro-inflammatory transcription factors such as TNF-α[5], IL-1β, and NF-κB, which leads to the expression of pro-inflammatory cytokines[6].

The brain and the immune system communicate through two different pathways. In the neural pathway, peripherally produced pathogen-associated molecular patterns (PAMPs), such as LPS, and cytokines activate primary afferent neurons, which project to the spinal cord and the nucleus of the solitary tract (NTS), from there to the parabrachial nucleus, ventrolateral medulla, the hypothalamic paraventricular and supraoptic nuclei,

the central amygdala, and the bed nucleus of the stria terminalis, which ultimately contributes to sickness behaviors. The humoral pathway involves circulating PAMPs that reach the brain at the level of the choroid plexus and the circumventricular organs, such as the area postrema. In the circumventricular organs, PAMPS induce production and release of pro-inflammatory cytokines[7].

The vagus nerve is involved with detecting LPS within the bloodstream and responding to inflammation. The cell bodies of vagal afferent fibers are located within the nodose ganglia, and there have been studies that have shown that TLR-4 receptor proteins and mRNA are present in the nodose ganglia based on protein detected by western blot[4]. Therefore, it is probable that LPS activates vagal afferent neurons through interaction with TLR-4[4]. In addition, experiments have shown through subdiaphragmatic vagotomy that an intact vagus nerve is necessary for LPS and cytokines to have their normal physiological effect[8].

The endocannabinoid system, which is made up of the cannabinoid receptors, endogenous ligands of those receptors, and the enzymes that aid in production and breakdown of these ligands, is known to play an anti-inflammatory role during states of systemic infection through the vagus nerve[9]. These ligands, or endocannabinoids, are tetrahydrocannabinol (THC)-like molecules that are both synthesized and secreted based on physiological demand. There are two receptors that they bind to, including the CB_1[10] and CB_2 receptors[11].

The CB_1 receptor is located mainly in the central nervous system, and it is a G-protein coupled receptor. It acts to inhibit adenylyl cyclase and ultimately inward rectifying K channels. This decreases cAMP concentration and activation of protein

kinase A[11]. This also inhibits the influx of calcium into the pre-synaptic neuron, which decreases neurotransmitter release through fusion of vesicles with the cell membrane[12]. Endocannabinoids are broken down by reuptake into the cells, movement into the appropriate cellular compartment, and fatty acid amide hydrolase (FAAH)-mediated hydrolysis[13].

Anandamide (AEA) and 2-arachidonoyl glycerol (2-AG) are the two most studied natural ligands for the CB receptors, and they have been shown to act as anti-inflammatory agents in the gastrointestinal tract. This has been shown through the use of an antagonist to increase inflammation and an agonist to decrease it in two different models of gastrointestinal inflammation in mice, one using dextrane sulphate sodium and another using intrarectal infusion of dinitrobenzene sulfonic acid[1]. When the amount of inflammation in the gut was assessed in a knock-out model for the CB_1 receptor, it was shown that there were higher levels of inflammation in knock outs compared to control animals[1,14]. There are more CB_1 receptors in the inflamed gut in a model induced using croton oil, indicating that there is an up-regulation during inflammation[15]. This implies that they are more active in inhibiting contraction during an inflammatory state. Also, mice are less susceptible to gastrointestinal inflammation when they are deficient in FAAH[9]. There is also a quicker endocannabinoid turnover rate in the inflamed gut[16]. All of these factors indicate that cannabinoids have an anti-inflammatory role in the gastrointestinal tract during a pathophysiological state.

Endocannabinoids, specifically 2-AG, have been shown experimentally to cause a decrease in phosphorylated NF-κB (pNF-κB), COX-2, p-ERK1/2, and p-p38MAPK expression after exposure to LPS for 12 hours in primary culture of caudate nucleus

neurons[17]. ERK, p38MAPK, and NF-κB all have involvement in the expression of COX-2 and other inflammatory mediators that are induced by LPS[18]. COX-2 causes prostaglandin synthesis and an increased synthesis of additional cytokines[19]. The effect of 2-AG in that study was shown to be through the CB_1 receptor[17]. CP 55,940 has also been shown to have an inhibitory effect on LPS-induced cytokine mRNA expression, including IL-1β, IL-6, and TNF-α, in rat cerebellar granule cells[20] and in rat microglial cells[21] through a CB receptor-independent mechanism. However, anandamide and 2-AG were unable to inhibit the inflammatory cytokine expression in these neurons, so the authors propose that CP 55,940 may inhibit cytokine expression by having an effect on reactive oxygen species-dependent activation of NF-κB independent of cannabinoid receptors, at least in cerebellar granule cells[20].

Since the vagus nerve is involved in gastrointestinal function, such as motility, it would be logical to propose that the potential antagonistic effect of cannabinoids on inflammation caused by LPS in the nervous system through vagal afferents would have downstream effects on gastrointestinal function, and this has been shown experimentally[22,23,24,5]. This may be very beneficial, because it could lead to potential therapies for widespread inflammation-induced gastrointestinal syndromes such as inflammatory bowel disease and irritable bowel syndrome. By showing that cannabinoids can decrease neuronal activation caused by LPS in vagal afferent neurons through action at the CB_1 receptor, the researchers could reveal potential nervous system targets for pharmacological therapies for the disorders previously mentioned as well as other autoimmune disorders.

The hypothesis is that cannabinoids decrease activation of nodose ganglion neurons induced by LPS indirectly through downstream effects. When LPS is released, there is an increase in activation as shown by an increase in cFos within the nucleus. Therefore, we propose that cannabinoid binding to CB_1 decreases the ability for LPS to activate vagal afferent neurons.

Materials and Methods

The animals in this study were adult male Sprague-Dawley rats (200-350g) from Harlan in Indianapolis, IN. They were housed at 22^{o}C with a 12:12 light:dark cycle and given access to food and water ad libitum. The protocol was approved by the University of Georgia Institutional Animal Care and Use Committee.

Extraction of Nodose Ganglia

Non-fasted rats were anesthetized using a cocktail containing ketamine (50mg/kg of body weight), acepromazine (3.3mg/kg of body weight), and xylazine (3.3mg/kg of body weight). The rat was positioned in dorsal recumbency and an approximately 4cm incision was made in the neck over the trachea. The vagus was located using blunt dissection techniques to separate the digastric, sternocleidomastoid, and sternothyroid muscles to identify the nerve coursing along the carotid artery. The nodose ganglia were identified and extracted. Once they were removed, they were placed in Hibernate solution (Brain Bits; Springfield, IL) on ice until they were used for cell culture.

Cell Isolation and Culture

Primary cells were isolated and cultured according to the procedure outlined in Simasko *et al*[25]. Both the left and the right ganglia from two different rats were combined in one isolation. The ganglia were transferred along with 2-3mL of Hibernate solution into a 35mm culture dish. The ganglia were then de-sheathed under a dissecting scope using sterile forceps and a 23-gauge needle. Then, they were transferred into enzyme digestion solution (3mg dispase, 3mg collagenase, 3mL Hank's Balanced Salt Solution without Calcium and Magnesium). The ganglia were minced using a scalpel and forceps. The dish was placed in an incubator (37ºC, 5% CO_2) for digestion for 90 minutes. Once the dish was removed, approximately half of the solution from the dish was pipetted into a 15mL conical centrifuge tube using a pipette coated with Sigmacote (Sigma-Aldrich; Saint Louis, MO). Then, using the same pipette, the ganglia were triturated in the dish. Following that, the ganglia were transferred into the centrifuge tube along with the rest of the solution in the culture dish. With a plain pipette, the culture dish previously containing enzyme digestion solution was filled with media made up of HEPES-buffered DMEM (HDMEM) supplemented with antibiotic (penicillin and streptomycin) and 10% fetal bovine serum (FBS) as to rinse the dish of any cells left. This media was transferred to the culture dish containing the minced ganglia. This was repeated until there was 10mL of media in the conical tube. Then, the tube was centrifuged at 140G for 1.5 minutes with no brake on. Once the tube was removed from the centrifuge, the supernatant was removed until 1.0mL was left in the bottom. With pipettes coated with Sigmacote, the pellet was re-suspended and more media was added until the total amount in the tube was 10mL. Centrifugation was repeated. Finally, with

another pipette coated with Sigmacote, the supernatant was removed once again until 1.0mL remained at the bottom. The pellet was re-suspended, and the solution was divided between six coverslips that had been pre-treated with 0.1mg/mL of poly-L-lysine hydrobromide (Sigma-Aldrich; Saint Louis, MO). The cells were placed in the incubator for approximately three hours, and then enough media was added to cover the slip and the slips were returned to the incubator. The following morning, the old media was removed and new media was added to each of the dishes.

Cell Treatment

The cells were incubated in HDMEM + 10% FBS for 72 hours before they were treated with either media (control), 1µM CP 55,940 (Tocris; Bristol, UK), 100pg/mL, 1ng/mL, 10ng/mL, or 20ng/mL *E. coli* O55:B5 LPS (List Biological Laboratories, Inc.; Campbell, CA), 0.3µM, 1µM, or 3.0µM CP 55,940 with 10ng/mL LPS, or 1µM AM251 (Tocris; Bristol, UK) with 1µM CP 55,940 and 10ng/mL LPS. When treated with just CP 55,940, the CP 55,940 was left on the cells for fifteen minutes, and then it was replaced with media for two hours. The LPS treatments were left on for two hours. The CP 55,940/LPS studies were performed by treating the cells with CP 55,940 for fifteen minutes, and then replacing the CP 55,940 with LPS for two hours. The AM/CP 55,940/LPS studies were conducted by combining the AM251 and CP 55,940 and treating the cells with the combined drugs for fifteen minutes, then replacing the solution with LPS for two hours. After treatment, the cells were fixed with 4% paraformaldehyde.

Immunohistochemistry

First, the slips were incubated in 0.3% H_2O_2 for an hour. Then, they were blocked in 4% normal goat serum for two hours. Two different antibodies were used in this experiment. The first antibody was rabbit polyclonal anti-cFos from Calbiochem (Darmstadt, Germany) (1:40,000), and the second antibody was mouse monoclonal anti-neurofilament heavy chain from Sigma-Aldrich (Saint Louis, MO) (1:500). This anti-neurofilament antibody recognizes both the phosphorylated and non-phosphorylated forms of neurofilament within culture. The cells were incubated in these antibodies together for approximately 72 hours. Following incubation, they were washed three times for twenty minutes in phosphate buffered saline (PBS) with Triton. Then, the protocol was followed from the rabbit Vectastain ABC Kit (Vector Laboratories, Inc; Burlingame, CA), and DAB peroxidase substrate (Vector Laboratories, Inc; Burlingame, CA) was used as a chromagen for the cFos staining. After the DAB was developed, the cells were washed three times for twenty minutes in PBS with Triton, and the mouse anti-goat AlexaFluor 488 from Molecular Probes (Invitrogen; Grand Island, NY) was used as a fluorescent antibody to visualize the neurons in culture. Primary and secondary antibody controls were done to ensure staining was specific.

Cell Counting

The total number of neurons stained with fluorescent secondary antibody to neurofilament was counted using a fluorescent Nikon UFX-IIA microscope. Those neurons that were also stained for cFos were counted as well. The researcher counting the cells was blinded to the treatment group of each coverslip. The percentage of cFos

positive neurons was calculated based on the number of neurons that had also been stained with cFos. These numbers were compared between different treatment groups [26].

Statistical Analysis

A one-way ANOVA test was used to determine whether the means of the treatment groups are significantly different when $p < 0.05$. For those treatments that were significantly different based on ANOVA, a two-tailed t-test was used to determine which specific treatments were significantly different from one another ($p < 0.05$). When appropriate, a two-way ANOVA test was performed to determine whether or not there was a significant interaction between two different variables.

Results

Activation of nodose ganglia neurons with LPS

The nodose ganglia neurons isolated were incubated for 72 hours before they were treated with LPS. The concentrations used were: control, 100pg, 1ng, 10ng, and 20ng/mL (n = 28, 9, 11, 25, and 10 coverslips respectively). The cells were stained for neurofilament and cFos (Figure 3-1). On average, there were 138 neurons per coverslip that stained positive for neurofilament as shown by immunofluorescence. Of the neurons that stained positive for neurofilament, 36% of the controls stained positive for cFos, indicating that these neurons had been activated. Fifty-three percent of the neurons that were treated with 100pg/mL LPS, 59% of them treated with 1ng/mL LPS, 60% treated with 10ng/mL LPS, and 71% treated with 20ng/mL also stained positive for cFos as

shown by DAB secondary staining (Figure 3-2). Single factor ANOVA (df = 4) shows that treatment with LPS has a significant effect on the percent of neurons activated ($p = 1.28x10^{-22}$). This indicates that increasing concentrations of LPS cause an increased amount of vagal afferent neuron activation.

Effect of neuron activation with CP 55,940, a cannabinoid receptor agonist[27]

Another treatment group was treated with 1μM CP 55,940 for fifteen minutes prior to being incubated with media for two hours (n = 15 coverslips). These cells were stained with neurofilament and cFos. There were 44 neurons on average that stained positive for neurofilament on these coverslips. Of these, 26% also stained positive for cFos (Figure 3-3). This number was significantly different from the percentage of neurons that stained positive for cFos under control conditions ($p = 4.16x10^{-4}$). Therefore, CP 55,940 by itself appeared to have an inhibitory effect on neuron activation.

Effect of pretreatment of neurons activated by LPS with CP 55,940

Nodose ganglia neurons were pre-treated with 0.3μM (n = 12 coverslips), 1μM (n = 21 coverslips), or 3.0μM (n = 12 coverslips) CP 55,940 for fifteen minutes prior to treatment with 10ng LPS for two hours. On average, there were 82 neurons per coverslip that stained positive for neurofilament as shown by immunofluorescence. Of these neurons, approximately 52%, 36%, and 38% of those neurons treated with 10ng/mL LPS after pretreatment with 0.3μM, 1μM, and 3.0μM CP 55,940 respectively showed cFos immunoreactivity compared to 60% without pretreatment with CP 55,940 (Figure 3-3). This shows a significant decrease in neuron activation with pretreatment by 0.3μM, 1μM,

and 3.0μM CP 55,940 ($p = 4.57x10^{-4}$, $1.83x10^{-16}$, and $1.53x10^{-12}$) compared to treatment with 10ng/mL LPS. Treatment with 1μM and 3.0μM CP 55,940 was not significantly different from control (p = 0.98 and 0.50), whereas treatment with 0.3μM CP 55,940 was ($p = 8.51x10^{-7}$). Two-factor ANOVA (df = 1) shows that treatment with LPS and pre-treatment with CP 55,940 have a significant effect on the percent of neurons activated and also that there is an interaction between those two variables ($p = 9.59x10^{-11}$). These data indicate that treatment with CP 55,940 decreases activation of vagal afferent fibers by LPS.

Effect of the cannabinoid receptor reverse agonist, AM251, on the reversal of activation with LPS by cannabinoid agonists

The last treatment group was then treated with a combination of 1μM AM251 and 1μM CP 55,940 for fifteen minutes and then treated with 10ng/mL LPS for two hours (n = 12 coverslips). AM251 has an approximately 4-fold greater affinity for the CB_1 receptor than CP 55,940[28]. Of the 72 neurons per coverslip on average that stained positive for neurofilament, an average of 60% also stained positive for cFos (Figure 3-3). This number was significantly different from control ($p = 7.39x10^{-10}$), but it was not significantly different from the percentage of neurons activated with LPS (p = 0.62). As an additive control, some neurons were treated solely with AM251 (n = 8 coverslips). On average, there were 125 neurons per coverslip that stained positive for neurofilament as shown by immunofluorescence. The average percentage of neurons activated by AM251 was 33% (Figure 3-3). This was not significantly different from control (p = 0.46), and it was significantly different from the AM/CP 55,940/LPS treatment ($p = 4.04x10^{-8}$). Two-

factor ANOVA (df = 1) shows that treatment with AM/CP 55,940/LPS and CP 55,940/LPS has a significant effect on the percent of neurons activated and that there is an interaction between those two variables ($p = 1.08x10^{-11}$). Therefore, by blocking binding of CP 55,940 to CB_1 receptors with AM251, the inhibition of neuronal activation was abolished. This indicates that the decrease in activation with LPS involved the CB_1 receptor.

Discussion

Based on the percentage of neurons activated within primary cell culture using cells obtained from the nodose ganglia, the hypothesis has been accepted. Treatment of vagal afferent neurons with LPS increases cFos immunoreactivity in correlation with concentration of LPS treatment. This suggests that LPS increases vagal afferent neuron activation. Therefore, we have shown that LPS can activate nodose ganglia neurons in culture. We have also shown that pretreatment with CP 55,940 decreased or reversed neuron activation in response to 10ng/mL LPS. Treatment with AM251 reversed the effect of CP 55,940. This suggests that the decrease in activation is through binding to the CB_1 receptor. These results indicate that there is some common pathway involved between cannabinoids and LPS and their effects on vagal afferent neurons. The exact manner by which they interact is currently unknown, but there are a couple of potential pathways that could be involved.

It has been shown experimentally by Du *et al* using hippocampal neurons that 2-AG suppresses phosphorylation of NF-κB[29], a pro-inflammatory transcription factor,

through inhibition of ERK/p38MAPK as well as COX-2 expression, which are two main markers for tissue inflammation in response to LPS challenge[18]. This has been shown to be mediated through expression of PPAR-γ. PPARs are receptors located within the nucleus that act to regulate gene expression for processes such as metabolism, cell differentiation, and inflammation, and PPAR-γ specifically is known to suppress pro-inflammatory genes after stimulation with an inflammatory factor. These effects on PPAR-γ expression and NF-κB phosphorylation were shown to be CB_1 receptor dependent[30]. LPS has been shown to reduce PPAR-γ expression[30]. It is possible that the effect seen here involves the ability for cannabinoids to decrease phosphorylation of NF-κB and stimulate PPAR-γ expression. There is also a possibility that microglia within primary culture have an effect on ERK phosphorylation, which could abolish nitric oxide release, resulting in neuroprotection[31]. Future studies will be done to elucidate whether these pathways are involved in the results seen with this experiment.

During inflammation, macrophages release pro-inflammatory cytokines, which communicate with the brain through vagal afferent fibers using mainly neural pathways. These cytokines lead to changes in neuronal function, which ultimately leads to sickness behavior if the physiological insult is large enough. Recently, inflammation has been implicated in many chronic illnesses, but inflammation is not necessarily a bad thing. In fact, inflammation is actually a defensive and protective method to fight infection. Also, sickness behaviors are thought to aid in survival by conserving energy that is needed to fight infection. These defense mechanisms do become a problem, though, when the response is not properly turned off.

When the response is not stopped once the infection has been cleared, chronic inflammation occurs, and it leads to a constant production of pro-inflammatory cytokines. This can damage blood vessels, pancreatic tissue, joint tissue, gut mucosa, and other tissues. This leads to many common diseases, including allergies, arthritis, atherosclerosis, diabetes, GI disorders including Crohn's disease and irritable bowel syndrome, obesity, autoimmune disorders, and others. In fact, chronic inflammation is also associated with aging itself. In addition, the process of inflammation requires energy, which exacerbates sickness behaviors and can even lead to depression. Those who are chronically ill, the elderly, and those undergoing chemotherapy often times continuously experience sickness behaviors associated with inflammation and some may become depressed. By discovering more about how cannabinoids act on vagal afferent neurons during a state of inflammation, more opportunities for therapeutic pharmacological therapy can be discovered.

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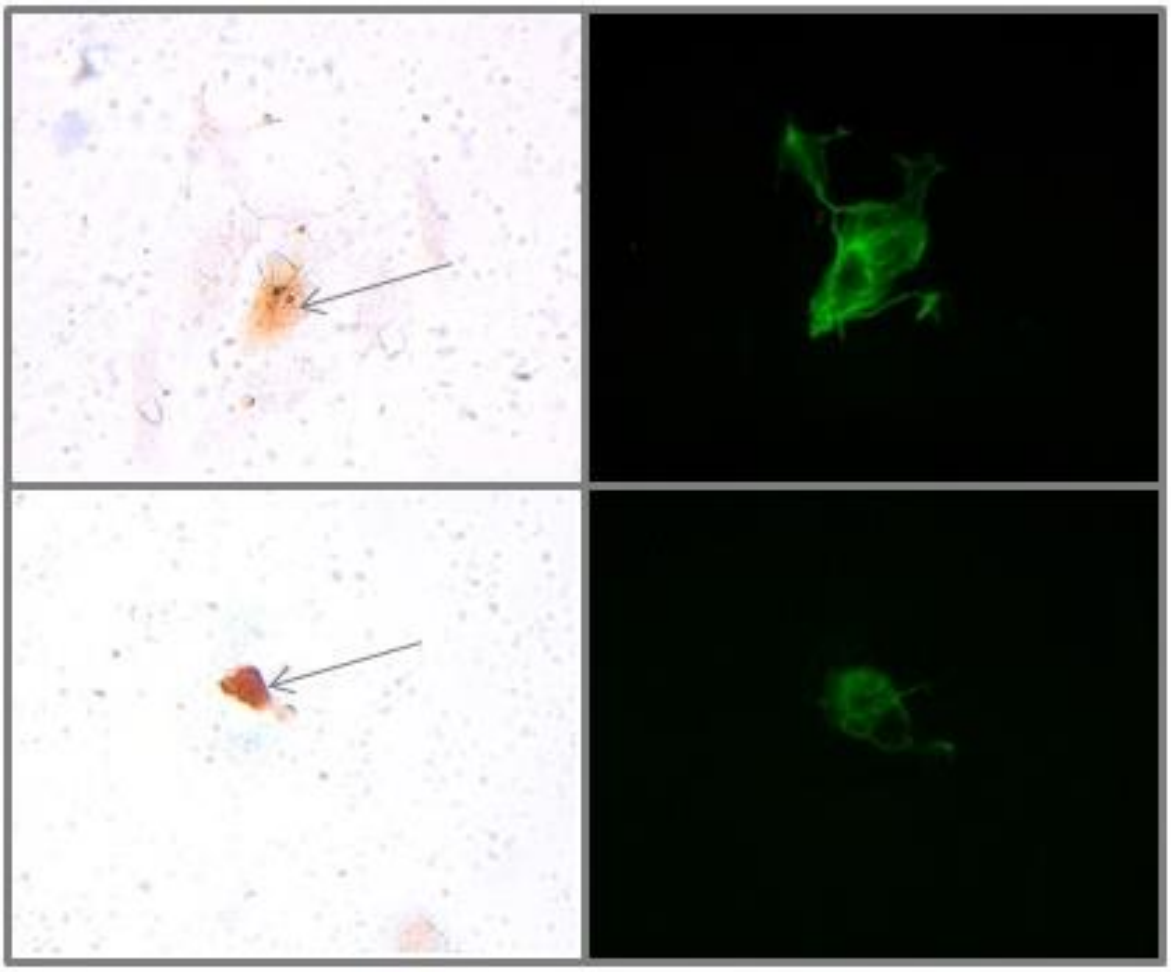

Figure 3-1. Neurons that have stained positive for neurofilament (right panels) and show either negative (top) or positive (bottom) cFos immunoreactivity (left panels).

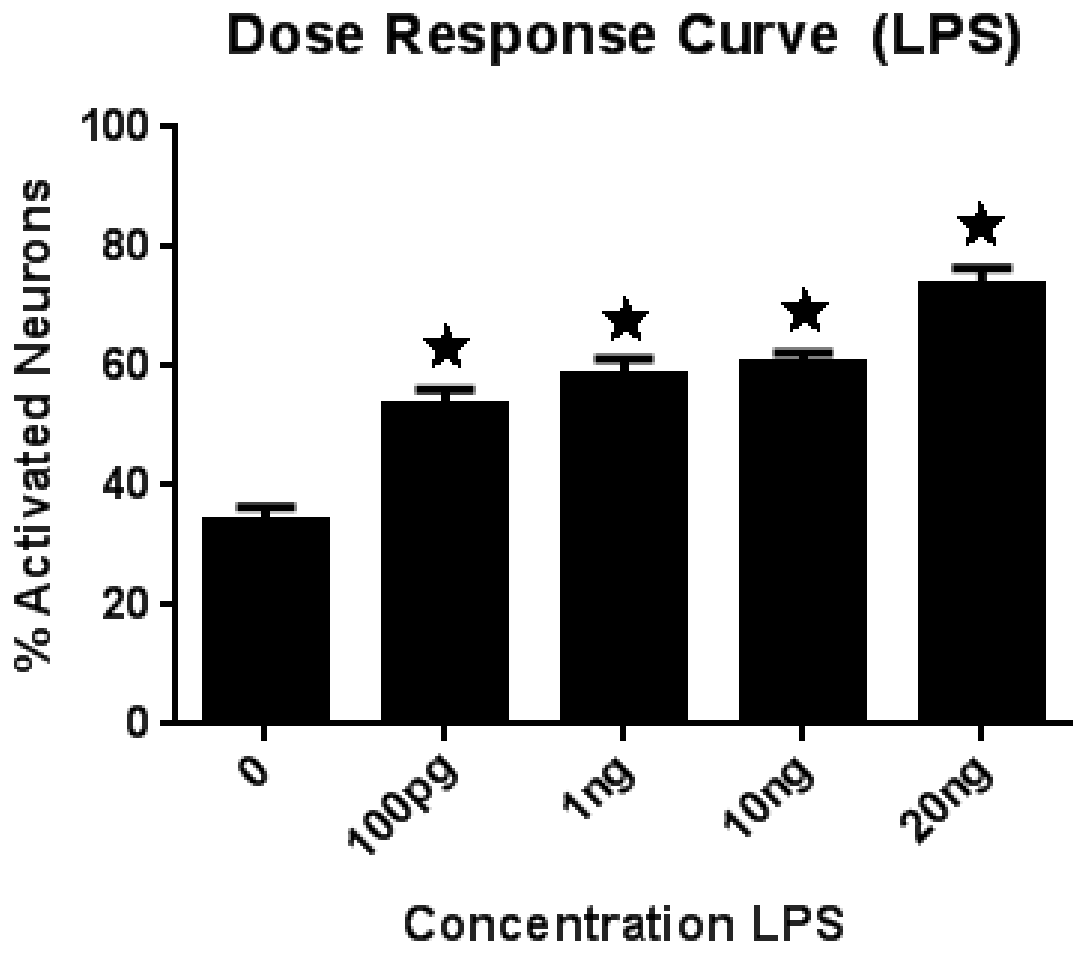

Figure 3-2. Percentage of neurons that are activated by control (n = 28 coverslips), 100pg/mL (n = 9 coverslips), 1ng/mL (n = 11 coverslips), 10ng/mL (n = 25 coverslips), and 20ng/mL (n = 12 coverslips) LPS. 36% of neurons are activated under control conditions. 100pg/mL LPS activates 53% of neurons on average from each coverslip, while 1ng/mL LPS activates 59% of neurons. 10ng/mL LPS activates 60%, and 20ng/mL LPS activates 71% of neurons. The percentage of activated neurons with 20ng/mL LPS was significantly different from all other treatments. *Significantly different from control ($p < 0.05$)

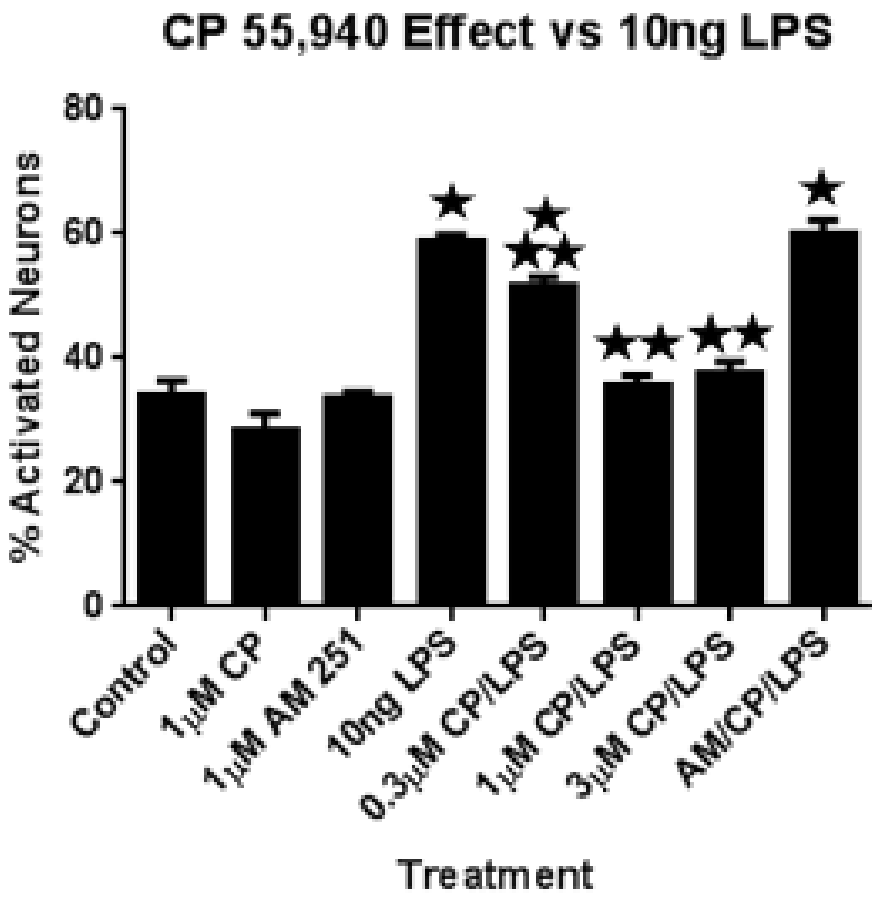

Figure 3-3. Percentage of neurons that are activated with 1μM CP 55,940 (n = 15 coverslips), 1μM AM251 (n = 8 coverslips), 10ng/mL LPS (n = 25 coverslips), 0.3μM (n = 12 coverslips), 1μM (n = 21 coverslips), and 3.0μM (n = 12 coverslips) CP 55,940/10ng LPS, and 1μM AM251/CP 55,940/LPS (n = 12 coverslips). Control = 36%, CP 55,940 (1μM) = 26%, AM251 (1μM) = 33%, LPS (10ng/mL) = 60%, 0.3μM CP 55,940/LPS = 52%, 1μM CP 55,940/LPS = 36%, 3.0μM CP 55,940/LPS = 38, and AM/CP 55,940/LPS = 60%. *Significantly different from control ($p < 0.05$). **Significantly different from LPS ($p < 0.05$).

CHAPTER 4

THE EFFECT OF CANNABINOIDS ON NF-KB AND SOCS-3 IN THE NODOSE GANGLIA AFTER ACTIVATION BY LPS [1]

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Abstract

Previous work has shown that cannabinoids decrease vagal afferent neuron activation that is caused by treatment with LPS through a mechanism involving the CB_1 receptor. TLR-4 receptors, which bind to LPS, and CB_1 receptors are both located on vagal afferent neurons. An interaction between signaling pathways involving LPS and cannabinoids has been suggested. The purpose of these studies is to explore the effects of pretreatment with a cannabinoid agonist, CP 55,940, on pNF-κB, NF-κB, and SOCS-3 expression induced by treatment with LPS in the nodose ganglia *in vitro.* To determine the effect of CP 55,940 and LPS on pNF-κB, NF-κB, and SOCS-3 expression, rats were anesthetized and nodose ganglia were extirpated. The ganglia were then desheathed and immediately treated with media, LPS, CP 55,940, CP 55,940 followed by LPS, AM 251, or AM 251 and CP 55,940 followed by LPS. Western blots were performed on the nodose ganglia in order to determine the relative expression of pNF-κB, NF-κB, and SOCS-3 after treatment. LPS treatment caused a decrease in expression of pNF-κB and NF-κB in nodose ganglia in addition to an increase in SOCS-3 expression. Pretreatment with CP 55,940 did not have an effect on pNF-κB or NF-κB expression in the nodose ganglia in response to LPS, but it did have a significant effect on SOCS-3 expression. This observation suggests that cannabinoids may have their effect on vagal afferent neuron activation through an indirect effect on signal transduction that most likely involves the production of cytokines.

Introduction

The vagus nerve is the tenth cranial nerve of the body, and it is involved in function of many organ systems, including the cardiovascular, pulmonary, and gastrointestinal systems[1]. It also plays a role in immune function by detecting pathogen associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) within the bloodstream and responding to resultant inflammation[2]. The brain and the immune system communicate and respond to PAMPs and inflammation through two different general pathways. In the neural pathway, PAMPs and cytokines activate primary vagal afferent neurons, which communicate to the brain through neural projections to the nucleus of the solitary tract (NTS) and other parts of the brain[3]. In the humoral pathway, PAMPs reach the brain at the level of the choroid plexus and circumventricular organs, such as the area postrema. In the circumventricular organs, PAMPs induce the production and release of pro-inflammatory cytokines[3].

Studies have shown that vagal afferent neurons have the capability to respond directly to the presence of LPS in the body through the expression of toll-like receptor 4 (TLR-4) in the nodose ganglia, where the cell bodies of vagal afferent neurons are located[2]. Other studies have shown that the vagus nerve not only has the capability to respond but also plays a large role in the body's response to inflammation. For example, experiments have shown that an intact vagus nerve is necessary for LPS and cytokines to have their normal physiological effect[4].

LPS causes inflammation by first binding to LPS binding protein (LBP) while circulating in the blood stream. LBP transfers LPS to a protein complex that is made up

of CD14, a GPI-anchored protein, the accessory protein MD-2, and heterodimerized TLR-4[2]. After binding to TLR-4, LPS activates MyD88, which recruits IL-1 receptor-associated kinase (IRAK) and TNF receptor associated factor (TRAF6). There have been studies conducted by Barbier de la Serre *et al* that indicate that there is an increase in TLR-4 activation in both ileal epithelium and vagal afferent neurons when these areas are under inflammatory stress due to an increase in LPS as shown through immunoreactivity of the TLR4/MD2 complex[5]. Recruitment of TRAF6 leads to the production of pro-inflammatory transcription factors such as NF-κB[6]. Before NF-κB is activated and translocated into the nucleus, it is bound to the inhibitor protein IκB within the cytoplasm. Activation of another molecule, IKKβ, causes IκB to become phosphorylated and eventually degraded, releasing NF-κB for translocation to the nucleus[7]. This nuclear translocation of NF-κB leads to the transcription of its target genes and the expression of pro-inflammatory cytokines[8] such as TNF-α[9], IL-1β, and IL-6[6]. These cytokines are responsible for causing the physiological symptoms associated with inflammation, including heat, swelling, redness, pain, and loss of function[6].

In order to ensure that LPS does not induce excessive inflammation, there is a negative feedback loop in place involving the TLR-4 receptor. The molecules produced through this negative feedback loop include suppressor of cytokine signaling-3 (SOCS-3). SOCS-3 expression is induced by the production of pro-inflammatory cytokines. Its anti-inflammatory function occurs by inhibiting the association between TRAF6 and TAK1, therefore causing inhibition of MAPK and NF-κB expression induced by LPS[10]. Therefore, when LPS binds to TLR-4, it causes the production of inflammatory cytokines

and also causes the production of molecules involved in anti-inflammatory processes such as SOCS-3.

This study looked at NF-κB and SOCS-3 signaling in particular and how this signaling is affected by both LPS and cannabinoids. There have been studies conducted showing that LPS binding to TLR-4 promotes translocation of NF-κB into the nucleus of vagal afferent neurons[11]. This may be a direct effect on vagal afferent neurons, because there is also evidence that TLR-4 and MyD88 are expressed on these neurons[11]. In addition, NF-κB signaling has been shown to induce SOCS-3 expression in the arcuate nucleus, and it is dose-dependently upregulated in cultured vagal afferent neurons in response to treatment with LPS[12]. There is also evidence that SOCS-3 expression is upregulated in response to LPS in macrophages and microglia[13,14].

The endocannabinoid system, which is made up of the cannabinoid receptors, endogenous ligands of those receptors, and the enzymes that aid in production and breakdown of these ligands, is known to play an anti-inflammatory role during states of systemic infection through the vagus nerve[15]. There are two receptors that they bind to, including the CB_1[16] and CB_2 receptors[15]. The CB_1 receptor is located mainly in the central nervous system, and it is coupled to a G-protein coupled receptor. It acts to inhibit adenylyl cyclase and ultimately inward rectifying K channels. This decreases cAMP concentration and activation of protein kinase A[15]. This also inhibits the influx of calcium into the pre-synaptic neuron, which decreases neurotransmitter release through fusion of vesicles with the cell membrane[17]. Endocannabinoids are broken down by reuptake into the cells, movement into the appropriate cellular compartment, and fatty acid amid hydrolase (FAAH)-mediated hydrolysis[18].

The two most studied natural ligands for the CB receptors, anandamide (AEA) and 2-arachidonoyl-glycerol (2-AG), have been shown to act as anti-inflammatory agents in the gastrointestinal tract in a number of studies. This has been shown through the use of an antagonist to increase gut inflammation and an agonist to decrease it in two different models of inflammation in mice[19]. There is also evidence that there is an increase in gastrointestinal inflammation in CB_1 receptor knock-out animals compared to control animals[19]. There is evidence of upregulation of the CB_1 receptor following induction of an inflammatory state in the gut using croton oil. In addition, mice are less susceptible to gastrointestinal inflammation when they are deficient in FAAH, the enzyme responsible for the breakdown of endocannabinoids[20]. All of these factors indicate that cannabinoids have an anti-inflammatory role in the gastrointestinal tract during a pathophysiological state.

Endocannabinoids, specifically 2-AG, cause a decrease in phosphorylated NF-κB (pNF-κB), COX-2, p-ERK1/2, and p-p38MAPK expression after exposure to LPS for 12 hours in primary culture of caudate nucleus neurons[21]. ERK, p38MAPK, and NF-κB all have involvement in the expression of COX-2 and other inflammatory mediators that are induced by LPS[22]. COX-2 causes prostaglandin synthesis and an increased synthesis of additional cytokines[23]. This effect was shown to be through the CB_1 receptor[21]. CP 55,940 has also been shown to have an inhibitory effect on LPS-induced cytokine mRNA expression, including IL-1β, IL-6, and TNF-α, in rat cerebellar granule cells[24] and in rat microglial cells[25] through a CB receptor-independent mechanism. However, anandamide and 2-AG were unable to inhibit the inflammatory cytokine expression in these neurons, so the authors proposed that CP 55,940 may inhibit cytokine expression by having an

effect on reactive oxygen species-dependent activation of NF-κB independent of cannabinoid receptors, at least in cerebellar granule cells[24].

Based on the observations previously outlined, we hypothesize that treatment with LPS will decrease the protein expression of pNF-κB and NF-κB and increase SOCS-3 expression as shown by an increase in concentration within nodose ganglia tissues. In addition, we hypothesize that treatment with CP 55,940, a cannabinoid receptor agonist[26], will reverse the effect of LPS on the expression of pNF-κB, NF-κB, and SOCS-3 in nodose ganglia.

Materials and Methods

The animals in this study were adult male Sprague-Dawley rats (200-350g) from Harlan in Indianapolis, IN. They were housed at 22^{o}C with a 12:12 light:dark cycle and given access to food and water ad libitum. The protocol was approved by the University of Georgia Institutional Animal Care and Use Committee.

Extraction of Nodose Ganglia

Non-fasted rats were anesthetized using a cocktail containing ketamine (50mg/kg of body weight), acepromazine (3.3mg/kg of body weight), and xylazine (3.3mg/kg of body weight). The rat was positioned in dorsal recumbency and an approximately 4cm incision was made in the neck over the trachea. The vagus was located using blunt dissection techniques to separate the digastric, sternocleidomastoid, and sternothyroid muscles to identify the nerve coursing along the carotid artery. The nodose ganglia were

identified and extracted. Once they were removed, they were placed in Hibernate solution (Brain Bits; Springfield, IL) on ice until treatment.

Cell Treatment

Two ganglia were pooled in a microcentrifuge tube and then treated with either media (control) for two hours, 10ng/mL *E. coli* O55:B5 LPS (List Biological Laboratories, Inc.; Campbell, CA) for two hours, 1µM CP 55,940 (Tocris; Bristol, UK) for fifteen minutes followed by media for two hours, 1µM CP 55,940 with 10ng/mL LPS, or 1µM AM251 (Tocris; Bristol, UK) with 1µM CP 55,940 and 10ng/mL LPS. The CP 55,940 and LPS treatments were performed by treating the cells with CP 55,940 for fifteen minutes and then replacing the CP 55,940 with LPS for two hours. The AM, CP 55,940, and LPS studies were conducted by combining the AM251 and CP 55,940 and treating the cells with the combined drugs for fifteen minutes, then replacing the solution with LPS for two hours.

Tissue Preparation

Following treatment, the samples were prepared for western blots. First, the cells were lysed using the following protocol. The ganglia were bathed in a suspension buffer made with 3.03g Tris, 12.7mL 0.5M EDTA, pH 8 in 500mL of water. The suspension buffer was fortified with 2.87µL of PMSF (10mg/mL alcohol), 1% Triton X 100, a 1% solution made with one cOmplete Mini, EDTA-free protease inhibitor cocktail tablet (Roche Diagnostics; Indianapolis, IN), and a 1% solution made with one PhosSTOP phosphatase inhibitor cocktail tablet (Roche Diagnostics; Indianapolis, IN) in order to

lyse the cells and properly measure and quantify the protein. Five microliters of solution was added per microgram of tissue. Typically, 10µg of tissue was obtained. The sample was homogenized using a pestle for approximately 10 seconds, and then the sample was sonicated on ice very briefly 5 times. Finally, the solution was aliquoted in 10µL increments and frozen at -80^{o}C until later use.

Protein Assay

In order to determine the amount of protein obtained per sample, the Bradford assay (Bio-Rad Laboratories, Inc.; Hercules, CA) was performed. The solution was mixed by adding 1 part reagent to 3 parts water, and the solution was filtered through a paper filter. Then, the standard curve was created using 1mg/mL IgG (Bio-Rad Laboratories, Inc.; Hercules, CA) to obtain 100µg/mL, 40µg/mL, 20µg/mL, 10µg/mL, 5µg/mL, 2µg/mL, and 1µg/mL solutions. Then, 3µL of Bradford reagent and 3µL of the standard curve or sample was combined. Lysis buffer was used as a blank. After five minutes, the absorbance at 595nm was read on an ND-1000 NanoDrop Spectrophotometer (ThermoFisher Scientific; Waltham, MA). Three replicates were used. The desired R^2 value was 0.99. Once that was obtained, the standard curve was used to determine the protein concentration in each sample.

Western Blotting

Tissue extracted from nodose ganglia was used, and 5µg or 10µg of protein was loaded. Sample buffer was prepared by mixing 950µL of 2X Laemmli sample buffer (Bio-Rad Laboratories, Inc.; Hercules, CA) with 50µL of beta-mercaptoethanol. The

protein sample was diluted 1:1 in sample buffer, and then the sample was heated at 95ºC for five minutes. Criterion TGX 4-20% pre-cast gels (Bio-Rad Laboratories, Inc.; Hercules, CA) were used for gel electrophoresis. Once the samples had been heated and the chamber was prepared, the samples and the Precision Plus Protein All Blue Standards ladder (Bio-Rad Laboratories; Hercules, CA) were loaded into the gel, and the gel was run for one hour at 200V.

Amersham Hybond-P PVDF membranes (GE Healthcare; Buckinghamshire, UK) were used for protein transfer. The proteins were transferred from the gel to the membrane for one hour at 100V. Once the proteins had been transferred to the membrane, 50% SeaBlock Blocking Buffer (Thermo Scientific; Rockford, IL) in TBS-Tween 20 (TBST) was used to block the membrane for one hour. Then, primary antibodies were left to incubate overnight. The following antibodies were used: 1:1000 pNF-κB, 1:1000 NF-κB, and 1:1000 SOCS-3 (Cell Signaling Technology; Danvers, MA) in 10% SeaBlock with TBST. Polyclonal rabbit GAPDH (1:10,000) was used as a loading control (Thermo Scientific; Rockford, IL). After overnight incubation, the membrane came to room temperature for an hour. After that, it was washed 6 times for 5 minutes with TBST. Then, the membrane was incubated in 1:1000 peroxidase-labelled anti-rabbit secondary (Vector Laboratories; Burlingame, CA) in 10% SeaBlock for one hour. Following that, the membrane was washed again 6 times for five minutes. Then, Luminato Classico Western HRP substrate (Millipore Corporation; Billerica, MA) was placed on the membrane for between three and five minutes. Amersham Hyperfilm ECL (GE Healthcare; Buckinghamshire, UK) was used to detect protein on the membrane. Once the film was developed, it was analyzed using Image J Software. In order to

perform a loading control, the membrane was reused by using Re-Blot Mild solution diluted to 1X (Millipore Corporation; Billerica, MA). The re-blot solution was placed on the membrane for 15 minutes, and then the membrane was blocked again with 50% SeaBlock in TBST for two five-minute washes. Following that, the GAPDH primary was added and the above steps were repeated.

Statistical Analysis

A one-way ANOVA test was used to determine whether the means of the treatment groups are significantly different when $p < 0.05$. For those treatments that were significantly different based on ANOVA, a two-tailed t-test was used to determine which specific treatments were significantly different from one another ($p < 0.05$).

Results

Effect of LPS on NF-κB, pNF-κB, and SOCS-3 expression in whole nodose ganglia

Protein expression of NF-κB and pNF-κB was measured by Western blot of whole nodose ganglia that were treated with 10ng/mL of LPS (n = 7 pairs of ganglia) or media (n = 7 pairs of ganglia) as a control (Figure 4-1). In control ganglia, there was a large amount of pNF-κB and NF-κB expression. However, as shown in Figures 4-2 and 4-3, there was a significant decrease in pNF-κB and NF-κB expression after treatment with LPS ($p = 0.030$, $p = 0.011$). The average adjusted density of control ganglia using GAPDH as a loading control was 1, whereas the average adjusted density of ganglia treated with LPS was 0.628 for pNF-κB and 0.657 for NF-κB. One-way ANOVA

showed a significant difference with treatment only for the expression of NF-κB ($p = 0.033$).

Protein expression of SOCS-3 was also measured by Western blot of whole nodose ganglia that were either treated with 10ng/mL of LPS (n = 10 pairs of ganglia) or media (n = 10 pairs of ganglia) as a control (Figure 4-4). In the control ganglia, there was little protein expression, but there was a significant increase in SOCS-3 expression after nodose ganglia were treated with LPS (Figure 4-5). The average adjusted density of control ganglia using GAPDH expression as a loading control was 1, whereas the average adjusted density of ganglia treated with LPS was 1.71. Single factor ANOVA (df = 1) shows that treatment with LPS has a significant effect on SOCS-3 expression in nodose ganglia ($p = 0.004$).

Effect of pretreatment of whole nodose ganglia with the cannabinoid receptor agonist CP 55,940 on pNF-κB, NF-κB, and SOCS-3 expression after activation by LPS

Whole nodose ganglia treated with CP 55,940 (1μM) for fifteen minutes prior to treatment with 10ng/mL LPS for two hours (n = 7 pairs of ganglia) revealed that there was no significant difference between the expression of pNF-κB in those ganglia and any other treatment groups (Figure 4-2). However, there was a significant decrease in NF-κB expression measured by Western blot between the control tissue, which had an average adjusted density of 1, and the tissue treated with CP and LPS ($p = 0.013$). Pretreatment with CP 55,940 did not have a significant difference on pNF-κB or NF-κB expression after treatment with LPS when compared to tissue that was solely treated with LPS ($p = 0.238$, $p = 0.493$) as seen in Figures 4-2 and 4-3. Single factor ANOVA (df = 3) did,

however, show a significant effect of treatment on NF-κB expression measured by Western blot ($p = 0.033$).

Whole nodose ganglia treated with CP 55,940 followed by LPS for two hours ($n = 5$ pairs of ganglia) did produce a significant difference between the expression of SOCS-3 in those ganglia and the ganglia treated with just LPS (Figure 4-5). There was a significant increase in SOCS-3 expression measured by Western blot between the control tissue, which had an average adjusted density of 1, and the tissue treated with just LPS, which had an average adjusted density of 1.71 ($p = 0.003$). In addition, there was a significant decrease in SOCS-3 expression induced by treatment with CP and LPS when compared to tissue that was solely treated with LPS ($p = 0.005$). Some tissue was treated solely with CP 55,940 as a control ($n = 3$ pairs of ganglia), and there was no significant difference between that tissue and the control tissue ($p = 0.43$). Single factor ANOVA ($df = 2$) did show a significant effect of treatment on SOCS-3 expression measured by Western blot ($p = 0.01$). However, most likely due to a small sample size of tissue treated with CP 55,940, two-way ANOVA ($df = 1$) did not show a significant interaction when comparing treatment groups with and without pretreatment with CP, 55940 ($p = 0.08$).

Effect of the cannabinoid receptor reverse agonist, AM251, and CP 55,940 on NF-κB, pNF-κB, and SOCS-3 expression

The last treatment group was treated with a combination of 1μM AM251 and 1μM CP 55,940 for fifteen minutes and then treated with 10ng/mL LPS for two hours ($n = 7$ pairs of ganglia). AM251 has an approximately 4-fold greater affinity for the CB_1

receptor than CP 55,940[27]. The average adjusted density of NF-κB after AM/CP/LPS treatment was 0.700 and that of pNF-κB after AM/CP/LPS treatment was 0.767 (Figures 4-2 and 4-3). The density was not significantly different from any other treatment groups for pNF-κB expression. However, NF-κB expression after AM/CP/LPS treatment was significantly different from control but not from any other treatment group ($p = 0.004$).

The average adjusted density of SOCS-3 after treatment with AM, CP, and LPS was also not significantly different from control ($p = 0.42$) as seen in Figure 4-5. This could be due to low sample size ($n = 2$ pairs of ganglia), which we are aiming to increase in the very near future.

Discussion

Our working hypothesis was that cannabinoids acting via the CB_1 receptor would increase NF-κB and decrease SOCS-3 expression in whole nodose ganglia induced by treatment with LPS. Our results indicate that LPS causes a decrease in NF-κB and pNF-κB expression in the nodose ganglia, but that the decrease is not reversed by pre-treatment with CP 55,940 as was hypothesized. However, LPS did cause an increase in SOCS-3 expression as expected based on past research, and CP 55,940 did reverse this observed increase[12-14].

Our data indicate that treatment of nodose ganglia tissue with LPS causes a decrease in both NF-κB and pNF-κB expression as well as an increase in SOCS-3 expression, which is in agreement with research conducted in other laboratories[11]. Once we established that treatment of nodose ganglia tissue with LPS *in vitro* can have an

effect on protein expression, we were able to demonstrate that pretreatment with CP 55,940 did not reverse the results of LPS treatment on NF-κB expression as expected, but it did reverse the effects on SOCS-3 expression. Previous data collected in this laboratory have shown that there is an increase in cFos staining following LPS treatment *in vitro* and that increase can be reversed by pretreatment with CP 55,940. This action was shown to be in response to action at the CB_1 receptor, because the activity of CP 55,940 on neuronal activation was reversed through treatment with AM251 (unpublished observation). These data led us to evaluate the molecular pathways by which these results were obtained. However, we found that CP 55,940 does not appear to have an effect on NF-κB or pNF-κB expression after treatment with LPS *in vitro* in nodose ganglia tissue but that it does have an effect on SOCS-3 expression.

There are two different pathways by which cannabinoids and LPS have been shown to interact (Figure 4-6). The researchers had originally proposed that it is most likely, given the past research done by a variety of different laboratories, that the resultant decrease in cFos activation seen with pretreatment with CP 55,940 involved NF-κB[21]. LPS binds to TLR-4, which recruits MyD88 and further activates IRAK and TRAF-6. TRAF-6 causes the translocation of NF-κB to the nucleus and increases phosphorylation of NF-κB[22]. Cannabinoids, on the other hand, have been shown to decrease the phosphorylation of NF-κB and to inhibit degradation of IκB, preventing translocation of NF-κB to the nucleus[21]. However, there is another pathway involving p38MAPK that could be responsible for the action that CP 55,940 has on vagal afferent neurons. Binding of a ligand to the CB_1 receptor causes inhibition of adenylyl cyclase, which down the line can lead to a decrease in phosphorylation of p38MAPK[28]. LPS binding to

TLR-4 has been shown to increase this phosphorylation. It would be prudent to discern whether or not this pathway is involved in the process in future studies. However, since SOCS-3 expression and NF-κB have been linked by previous studies, it is possible that NF-κB is still involved in the interaction previously shown[13].

It is possible that these results are different than results obtained by other researchers due to differences in tissue preparation and the area of interest. Many researchers that have done studies regarding cannabinoids and LPS have treated the animals *in vivo* and later extracted the tissues for analysis or have done the studies in culture rather than using whole ganglia[21,24,25]. In addition, many studies have been conducted in other parts of the brain, but they have not necessarily involved vagal afferent neurons and the nodose ganglia[21,24,25]. The lack of effect of cannabinoid pretreatment on NF-κB expression after treatment with LPS could also be due to the very high level of protein expression that was obtained. There was a significant difference observed with SOCS-3 expression, and there was less observable protein on the blot. The decrease in observable protein may have made subtle differences in expression more apparent. Future studies may evaluate the role of MAPK in the interaction between LPS and cannabinoid treatment, or the study could be repeated in cell culture or after *in vivo* treatment to see if the other studies conducted are repeatable in the nodose ganglia.

During an inflammatory state, macrophages release pro-inflammatory cytokines, which communicate with the brain through vagal afferent fibers using both neural and humoral pathways[3]. These cytokines lead to changes in neuronal function, which ultimately lead to sickness behavior if the physiological insult is large enough. When the response is not stopped once the infection has been cleared, chronic inflammation occurs,

and it leads to a constant production of pro-inflammatory cytokines[3]. This can damage blood vessels, pancreatic tissue, joint tissue, gut mucosa, and other tissues. This leads to many common diseases, including allergies, arthritis, atherosclerosis, diabetes, GI disorders including Crohn's disease and irritable bowel syndrome, obesity, autoimmune disorders, and others. In fact, chronic inflammation is also associated with aging itself[29]. Those who are chronically ill, the elderly, and those undergoing chemotherapy often times continuously experience sickness behaviors associated with inflammation and some may become depressed. By discovering more about how cannabinoids act on whole nodose ganglia and vagal afferent neurons specifically during a state of inflammation, more opportunities for therapeutic pharmacological therapy can be discovered.

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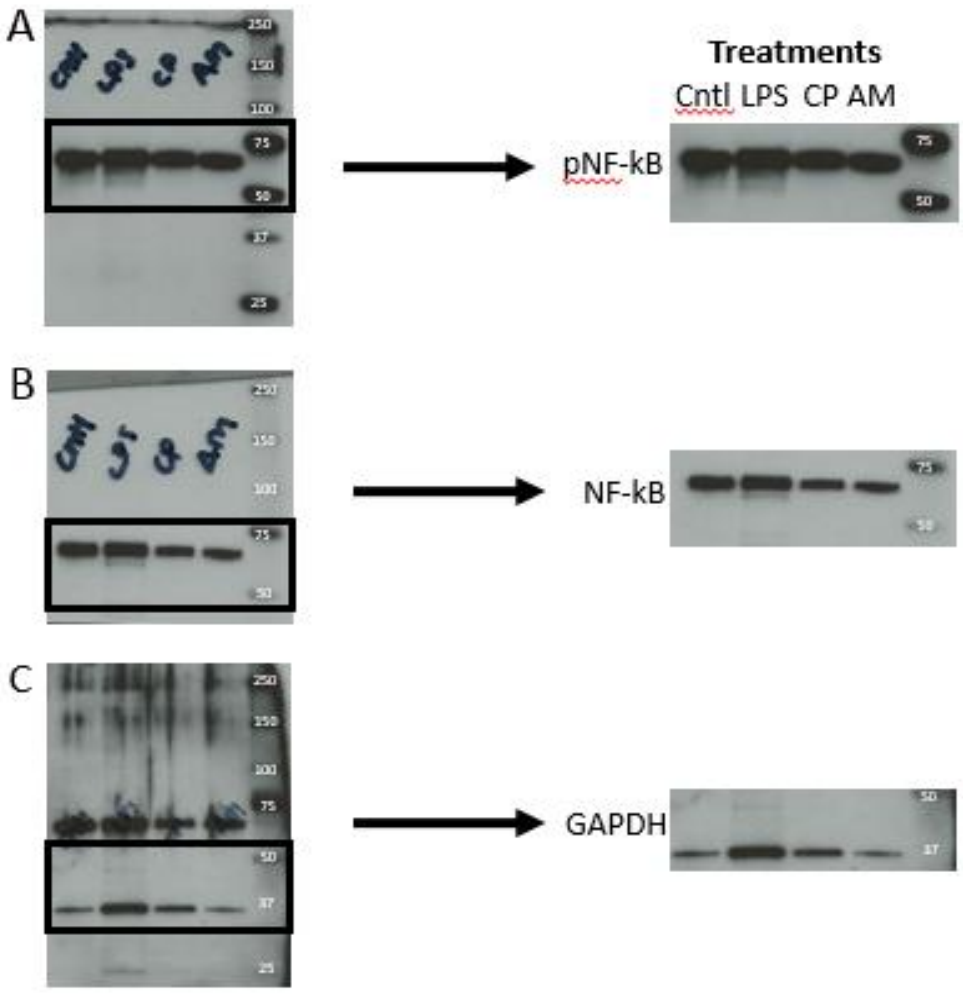

Figure 4-1. A. This is a representative Western blot incubated in 1:1000 pNF-κB primary antibody demonstrating the band corresponding with pNF-κB protein expression at 65kDa in nodose ganglia after treatment with media, 10ng/mL of LPS, 1μM CP 55,940 followed by LPS, or 1μM AM 251 and CP 55,940 followed by LPS. B. This is a representative Western blot incubated in 1:1000 NF-κB primary antibody demonstrating the band corresponding with NF-κB protein expression at 65kDa in nodose ganglia after treatment. C. This is a representative Western blot incubated in 1:10,000 GAPDH primary antibody demonstrating the band corresponding with GAPDH expression at 36kDa in nodose ganglia after treatment. GAPDH was used as a loading control.

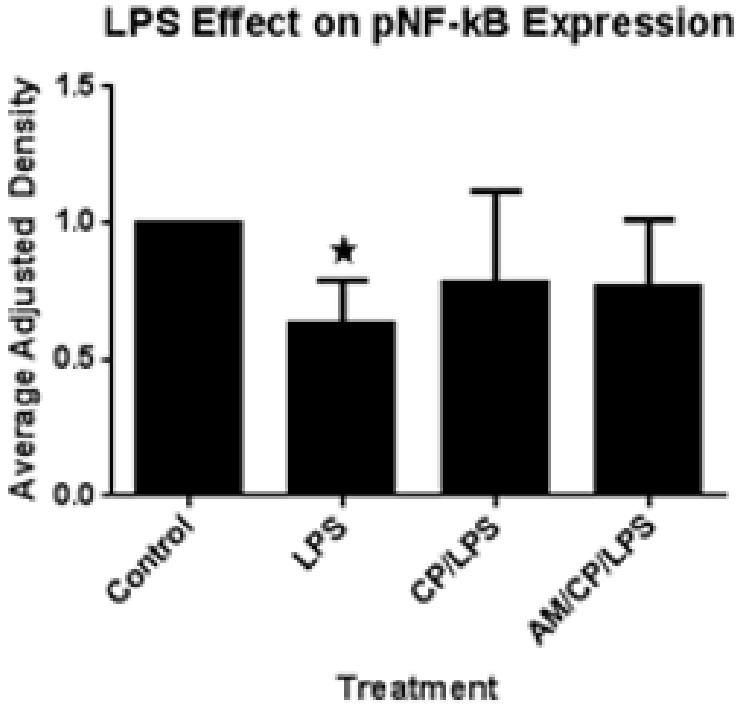

Figure 4-2. This graph represents the quantification of Western blots showing pNF-κB protein expression. There was a significant decrease ($p = 0.03$) in pNF-κB expression in the nodose ganglia after treatment with LPS. The average adjusted density for the expression of pNF-κB was set at 1 for the control group ($n = 7$ pairs of ganglia), and it was calculated at 0.628, 0.781, and 0.767 for LPS ($n = 7$ pairs), CP/LPS ($n = 7$ pairs), and AM/CP/LPS ($n = 7$ pairs) treatments respectively. * Significantly different from control ($p < 0.05$).

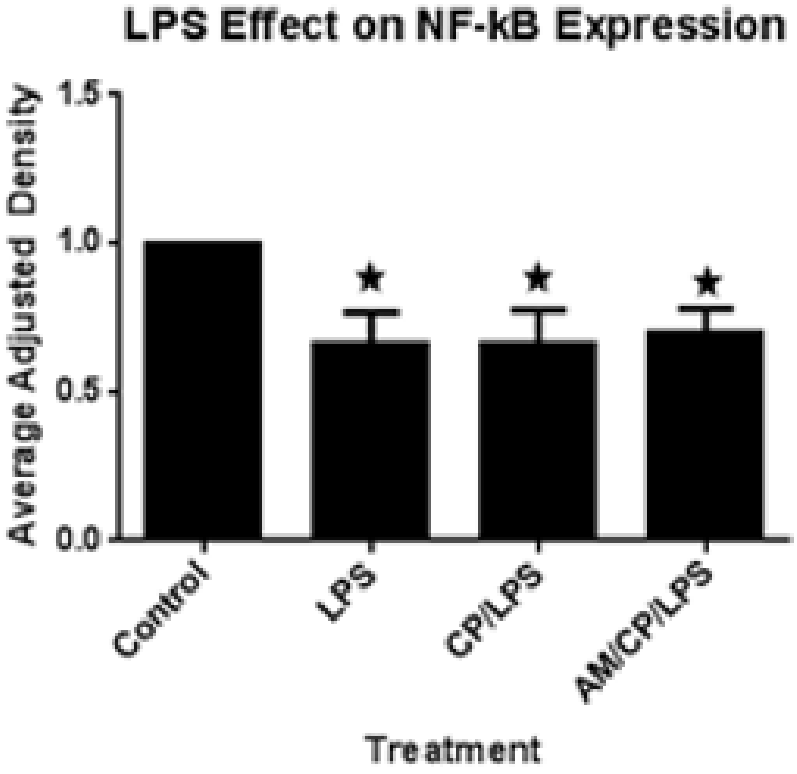

Figure 4-3. This graph represents quantification of Western blots showing NF-κB protein expression. There was a significant decrease in NF-κB protein expression between control and LPS, CP/LPS, and AM/CP/LPS ($p = 0.01$, $p = 0.01$, and $p = 0.004$). The average adjusted density for the expression of NF-κB was set at 1 for the control group ($n = 7$ pairs of ganglia), and it was calculated at 0.657, 0.659, and 0.700 for LPS ($n = 7$ pairs), CP/LPS ($n = 7$ pairs), and AM/CP/LPS ($n = 7$ pairs) treatments respectively. * Significantly different from control ($p < 0.05$).

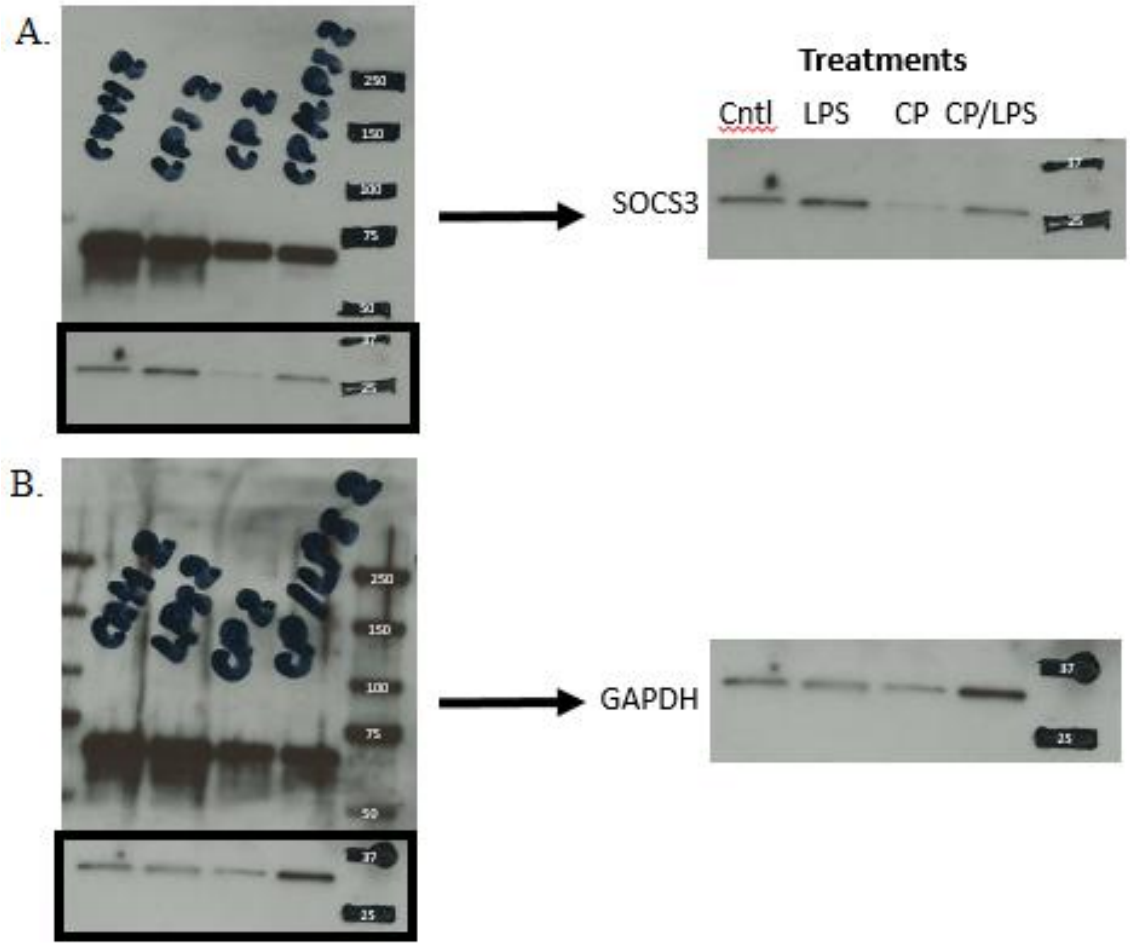

Figure 4-4. A. This image is a representative Western blot incubated in 1:1000 SOCS-3 primary antibody demonstrating the band corresponding with SOCS-3 protein expression at 26kDa in nodose ganglia after treatment with media, 1µM CP 55,940, 10ng/mL of LPS, 1µM CP 55,940 followed by LPS, or 1µM AM 251 and CP 55,940 followed by LPS. B. This image is a representative Western blot incubated in 1:10,000 GAPDH primary antibody demonstrating the band corresponding with GAPDH expression at 36kDa in nodose ganglia after treatment. GAPDH was used as a loading control.

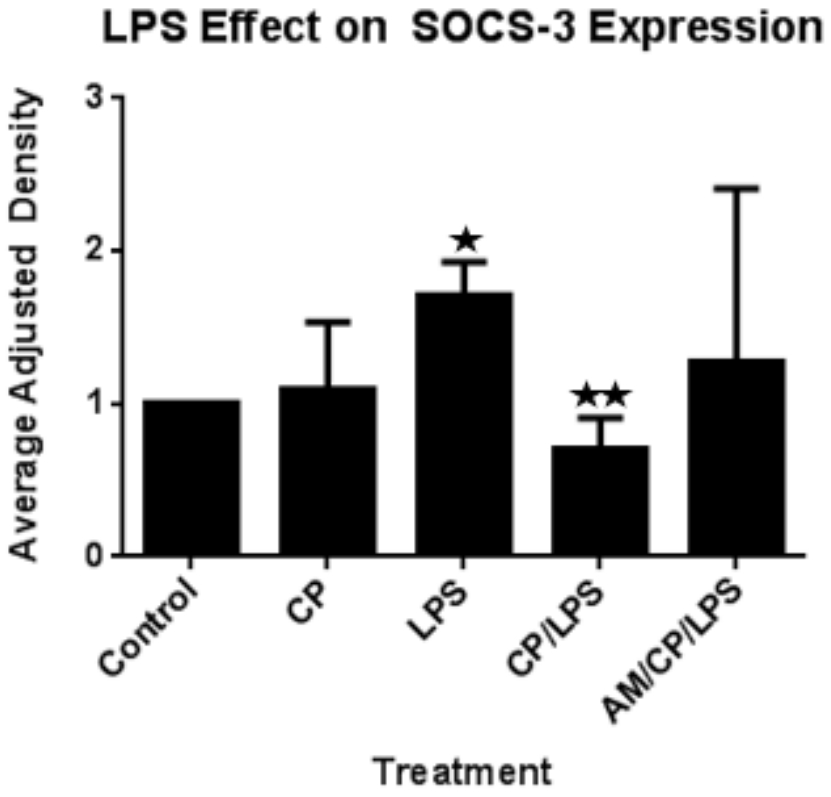

Figure 4-5. This graph represents the quantification of Western blots showing SOCS-3 protein expression. There was a significant increase ($p = 0.004$) in SOCS-3 expression in the nodose ganglia after treatment with LPS, and there was a significant decrease in expression with CP/LPS treatment when compared to treatment with LPS ($p = 0.03$). The average adjusted density for the expression of SOCS-3 was set at 1 for the control group ($n = 10$ pairs of ganglia), and it was calculated at 1.09, 1.71, 0.71, and 1.28 for CP ($n = 3$ pairs), LPS ($n = 10$ pairs), CP/LPS ($n = 5$ pairs), and AM/CP/LPS ($n = 2$ pairs) treatments respectively. * Significantly different from control ($p < 0.05$). ** Significantly different from LPS ($p < 0.05$).

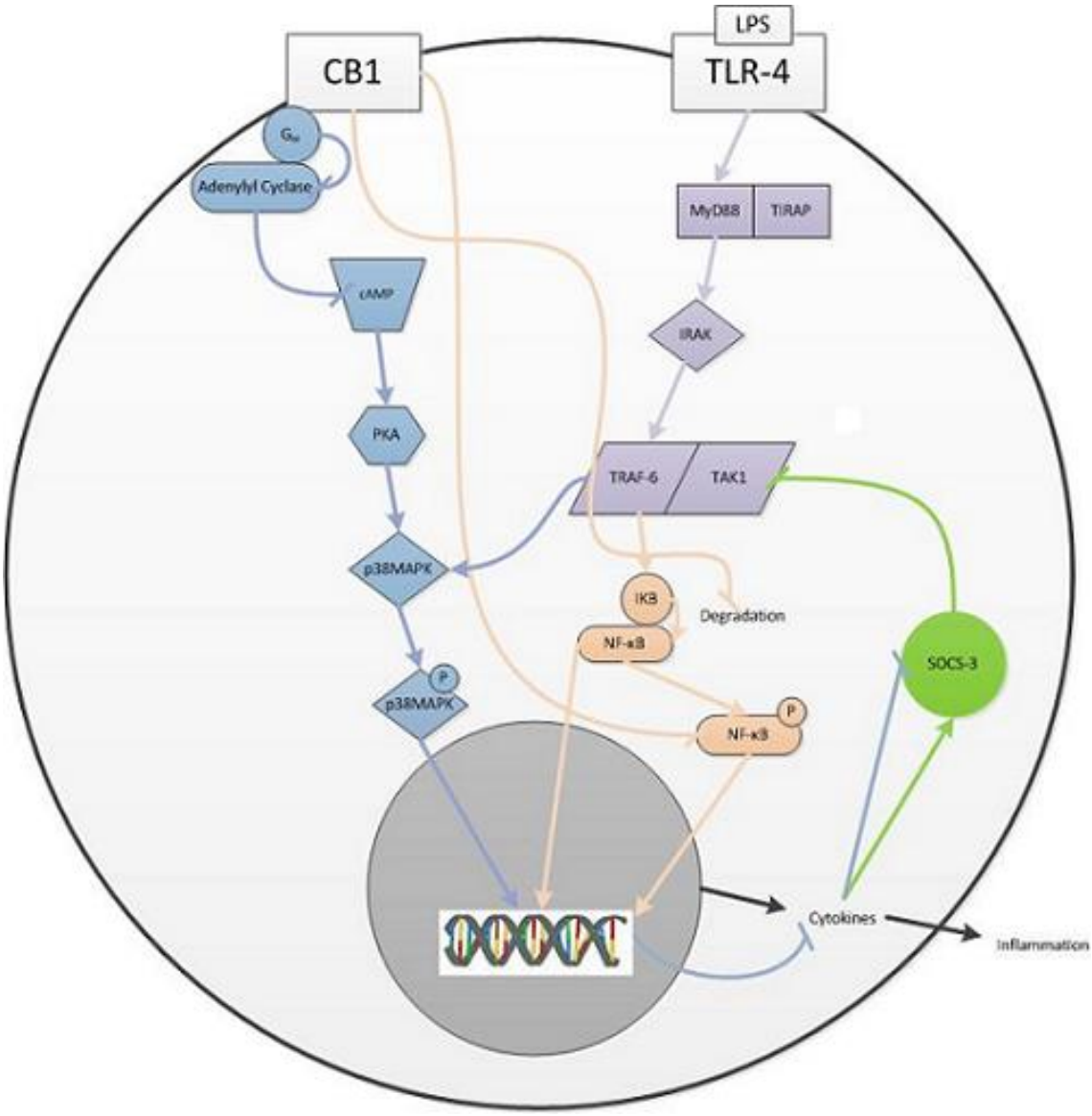

Figure 4-6. There are two different pathways through which LPS and cannabinoids may interact. One pathway involves phosphorylation of p38MAPK due to LPS activation of TRAF-6 and a decrease in phosphorylation of p38MAPK through inhibition of adenylyl cyclase by cannabinoids (blue pathway). The second pathway involves nuclear translocation and phosphorylation of NF-κB by LPS binding to TLR-4 and the inhibition of IkB degradation and NF-κB phosphorylation by cannabinoids (pink pathway). The purple pathway involved with the TLR-4 receptor is common to both the MAPK and NF-

κB pathways. SOCS-3 expression is induced by cytokine production and inhibits the association of TAK1 and TRAF6 (green pathway). Activation of the CB_1 receptor may decrease cytokine production, which would in turn decrease SOCS-3 expression.

CHAPTER 5

CONCLUSION

The vagus nerve innervates many different organs, and this innervation is integral in normal, everyday function involving the respiratory, cardiovascular, and gastrointestinal systems[1]. There are afferent, efferent, and central pathways that communicate with each other to sense stimuli and to cause appropriate reactions to those stimuli[2]. Many different neuropeptides are used to control satiation and appetite, and this usually involves the vagus nerve[3]. Cannabinoids play an important role in many aspects of gastrointestinal function, appetite, and during infection and inflammation. Cannabinoids could potentially be used in a therapeutic capacity in response to illnesses resulting in gastrointestinal dysfunction, such as inflammatory bowel disease[4]. These possibilities are important not only within the human population, but also in the pet population.

Initially, these studies showed through immunohistochemistry that vagal afferent neurons are activated in a dose dependent manner by CCK and that pretreatment with a cannabinoid agonist can decrease vagal afferent neuron activation after challenge with CCK in culture. This indicates that CCK and cannabinoids have the opposite effect on vagal afferent activation, which is also indicated by the physiological effects that each of these stimuli have on the gastrointestinal system – namely that CCK signals satiety and that cannabinoids signal hunger and food-seeking behaviors[2].

Next, these studies showed through immunohistochemistry that vagal afferent neurons are activated in a concentration dependent manner by LPS as well and that pretreatment with a cannabinoid agonist can decrease vagal afferent neuron activation after challenge with LPS in culture. This indicates that cannabinoids may have an anti-inflammatory effect on nodose ganglia neurons *in vitro* through a CB_1 receptor-dependent mechanism.

Lastly, these studies showed that LPS can decrease pNF-κB and NF-κB and increase SOCS-3 expression using Western blot in whole nodose ganglia. However, there was not a significant difference in pNF-κB or NF-κB expression with pretreatment with CP 55,940 following LPS challenge. This could have been due to the high protein expression found in every sample measured, but it may also indicate that the effects that cannabinoids have on neuronal activation within the nodose ganglia are independent of the NF-κB pathway. For example, they may involve p38MAPK, and further studies are necessary to determine if that is the case. However, CP 55,940 did attenuate the change in SOCS-3 expression observed after LPS treatment. This seems to indicate that CP 55,940 does at least have an indirect effect on cytokine expression and inflammation in nodose ganglia.

A number of different laboratories have come to conclude that, "evolution has integrated some regulatory pathways that govern the body's reactions to both pathogens and nutrition"[5]. This dissertation has gone on to further look into this conclusion by relating both of these stimuli, pathogens and nutrition, to the cannabinoid system. Obesity has recently been characterized as causing a low level of metabolic stress that leads to inflammation and an increase in LPS due to a change in gut microbiota[6].

Another laboratory has conjectured that "metabolic inflammation could also promote energy imbalance and obesity by interrupting regulatory signaling in the CNS"[5]. There is also evidence that the presence of NF-κB in peripheral tissues can affect glucose and lipid metabolism, which can in turn affect the release of CCK in those tissues[5]. Both of these studies seem to infer that there is involvement between CCK and other orexigenic and anorexigenic factors and LPS and inflammation, but that lends the question – where do cannabinoids fit into this picture?

CCK decreases food intake in response to luminal fat and protein by increasing CART and Y2 receptor expression through a PKC-dependent pathway[6]. Cannabinoids, on the other hand, increase food intake in response to fasting[7]. As these studies have shown, CCK and cannabinoid receptor activation are related to each other and they may even counteract the degree of hypophagia that is observed in sickness behaviors. LPS also decreases food intake during infectious inflammatory states due to activation of the hypothalamic-pituitary-adrenal axis and the production of cytokines[8]. In addition to this, CCK has been shown in a number of different tissues to either have an anti-inflammatory[9,10] or an inflammatory[11] effect on cells based on their location within the body. It has yet to be determined, however, whether activation of the CCK-1 receptor is inflammatory or anti-inflammatory within vagal afferent neurons.

I propose that there are two possible mechanisms by which all three of these receptors may interact (Figure 5-1), although it is very important to keep in mind that physiological mechanisms and signal transduction pathways are very convoluted and involved and this is just a proposed model. They could either act through p38MAPK, NF-κB transcription, or both[12,13]. The first step to see if this is even possible is to

determine whether or not the CCK-1, CB_1, and TLR-4 receptors are all colocalized within the same vagal afferent neuron.

The most important question to ask, however, is how could all of this be related? I propose that it is possible that they could all be related to the obesity epidemic which affects not only the human but also the pet population. Obesity has been shown to lead to an increase in LPS due to changes in gut microbiota, which further leads to an increase in chronic inflammation[6]. Obesity also "locks" the phenotype of vagal afferent neurons into a state resembling that of fasted control animals, namely there is an increase in CB_1 and MCH1 receptor expression and a decrease in Y2 and leptin receptor expression[14]. In addition, obesity results in insensitivity to CCK, 5HT, and leptin signaling as well as an insensitivity to gastric distention[14]. So, obesity causes an increase in orexigenic stimuli, a decrease in anorexigenic stimuli, and an increase in inflammation. Therefore, it would be interesting to see how all three – CCK, cannabinoids, and LPS – affect each other after acknowledging that all of them have effects on chronic and systemic inflammation, which can be induced by the state of obesity.

There are many ways in which to follow up these studies. One of the experiments that could be done to further shed light on the signaling pathways involved with CB_1 receptors and LPS is to determine whether or not the CB_1 receptor and TLR-4 are colocalized on the same neurons. This will help by narrowing down the number of secondary signaling pathways that may be involved with the interaction. In addition, there have been no experiments to date that have examined the presence of TLR-4 receptors on vagal afferent terminals. The main future path to investigate involves molecular experiments to look downstream of the receptors to see what secondary

signaling mechanisms are used and how this decrease in activation that has been shown occurs. More specifically, since other studies have shown that LPS can cause an increase in pNF-κB and cannabinoids can block that increase[15-17], it will be helpful to repeat the study involving NF-κB with cultured nodose ganglia neurons. Also, it would be interesting to see if there is an effect on COX-2 and/or MAPK expression to further illuminate the signaling mechanisms involved. This will aid in development of therapies using cannabinoids for sickness behavior by pin pointing the downstream target in order to avoid the dysphoria that is often associated with activation of the CB_1 receptor. In conclusion, there are many different ways that this experiment can be continued in order to discover more about the ant-inflammatory properties of cannabinoids and their effects on activation of vagal afferent neurons using CCK and LPS.

In summary, these studies have illuminated the effects that cannabinoids have on vagal afferent neuron activation after challenge with CCK and LPS as well as the effect that LPS has on NF-κB, pNF-κB, and SOCS-3 expression. The potential for therapeutic benefit from cannabinoids is significant. There are many different instances when increasing appetite and decreasing inflammation are beneficial. The elderly, those undergoing chemotherapy, and the chronically ill all experience a decrease in appetite, which leads to a decrease in energy intake. Energy is required for appropriate immune and inflammatory responses to fight infection and allow regeneration of damaged tissue. Identifying peripheral targets for cannabinoid action is important as these targets may provide a means of allowing beneficial effects of the cannabinoid while avoiding the central neural effects, such as dysphoria.

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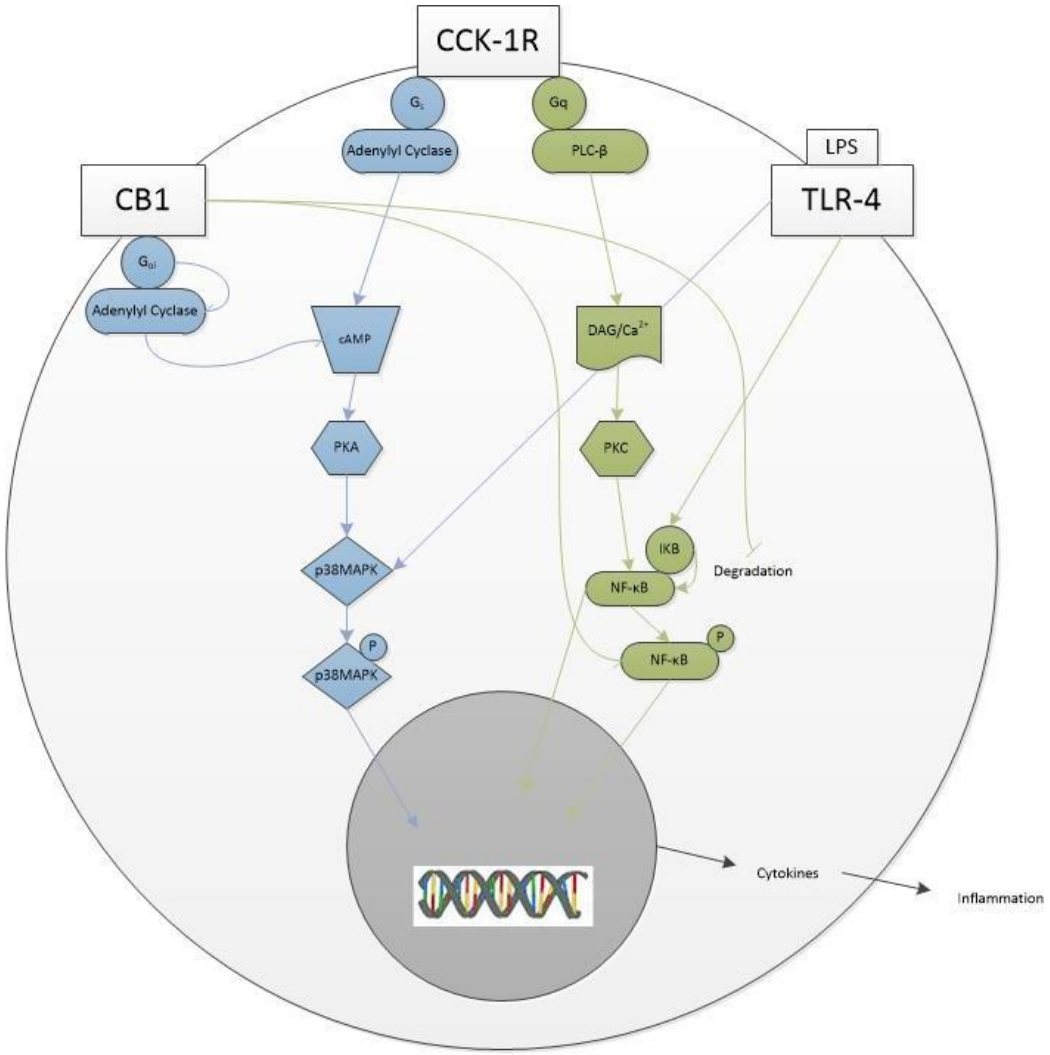

Figure 5-1. The proposal for how CB_1, CCK-1, and TLR-4 receptors may interact through signal transduction mechanisms. CB_1 receptors decrease cAMP concentration by inhibiting adenylyl cyclase, which decreases phosphorylation of p38MAPK, while TLR-4 and CCK-1 receptor activation causes phosphorylation of p38MAPK (blue). In addition, CB_1 receptors can inhibit degradation of IκB or decrease phosphorylation of NF-κB to prevent translocation of NF-κB into the nucleus of the neuron or decrease its efficacy. CCK-1 and TLR-4 receptors, on the other hand, can lead to an increase in the translocation of NF-κB and production of inflammatory cytokines (green).