

**INTERACTION OF CHOLECYSTOKININ AND CANNABINOID AGONIST CP55,940
IN THE NUCLEUS OF SOLITARY TRACT (NTS) AND AREA POSTREMA (AP) IN
REGULATION OF FOOD INTAKE**

by

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(Under the Direction of Gaylen L. Edwards)

ABSTRACT

The nucleus of the solitary tract (NTS), and area postrema (AP) located in the dorso-medial brain stem are major regions of the hindbrain important in the short term regulation of food intake. Cholecystokinin (CCK) is a well-studied satiety signal that plays important roles in the regulation of short-term food intake. Cannabinoids have been known for ages to increase the consumption of highly palatable foods. Studies from our lab have earlier have shown that intracerebroventricular (ICV) administration of cannabinoid agonist, CP55,940 into the 4th ventricle increased the consumption of sweetened condensed milk. Neurons in the NTS and AP are shown to express c-Fos protein following intraperitoneal (IP) injection of CCK. Our hypothesis is that cannabinoids decrease the activation of c-Fos in the neurons of NTS and AP by CCK and this effect involves catecholaminergic neurons. Cannulated rats were given CP55,940 (0.5µg/5µl/animal) via the 4th ventricle and were administered CCK (5µg/kgB.Wt, IP), 15 minutes after CP55,940 injection. Our results from this study showed that CP55,940 suppresses the c-Fos expression activated by CCK. The c-Fos counts in the CP/CCK treated rats, (NTS 69 ± 19, AP 30 ± 6; mean±SEM) were significantly lower than the counts in the saline/CCK treated group (NTS 371 ± 94; AP 150 ± 47; mean±SEM) (P<0.05). Results using the cannabinoid 1

receptor (CB1 receptor) antagonist SR141716, CP55,940 and CCK showed that the inhibitory effect of CP55,940 on c-Fos activation of CCK was effectively blocked using the SR141716. The c-Fos counts in the SR/CP/CCK treated rats were significantly higher than those from rats treated with saline/CP/CCK (104.3 ± 40.12 vs 24.29 ± 6.08). Double labeling studies using c-Fos and TH antibodies showed c-Fos expression activated by CCK in TH positive cells but there was no statistically significant difference in the number of TH positive neurons showing the c-Fos immunoreactivity between the different treatment groups. The number of TH positive neurons showing c-Fos immunoreactivity in CP/CCK treated rats, (NTS 39 ± 6 ; mean \pm SEM) were not significantly different than the counts in the saline/CCK treated rats (NTS 42 ± 9 ; mean \pm SEM) ($P > 0.05$). Similarly the counts of TH positive neurons showing c-Fos immunoreactivity in the NTS of SR/CP/CCK treated rats (13 ± 1) were not significantly different from the rats treated with saline/CP/CCK (7 ± 1), SR/saline/CCK (13 ± 1), saline/saline CCK (15 ± 4). These studies indicate that CCK induced c-Fos like immunoreactivity in the NTS and the activation of NTS neurons by CCK is inhibited by CP55,940. Our study also showed that cannabinoid antagonist SR141716 decreased the inhibitory effect of CP55,940 on neuronal activation of CCK suggesting that this effect is mediated through the CB1 receptor. Doublelabeling immunohistochemistry shows that this interaction of CCK and CP55,940 seems not to involve the catecholaminergic neurons in NTS.

INDEX WORDS: Cholecystokinin (CCK), CP 55,940, SR141716, NTS, AP

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Introduction

Proper body weight regulation is necessary for maintenance of good health. The taste and availability of food is related to the amount of food consumed and can contribute to overeating leading to obesity. Obesity is defined as having a very high amount of body fat in relation to lean body mass, or Body Mass Index (BMI) of 30 or higher. Body Mass Index (BMI) is a measure of an adult's weight in relation to his or her height, specifically the adult's weight in kilograms divided by the square of his or her height in meters. It is well known that unhealthy maintenance of body weight is a widespread problem, especially in the developed countries. Epidemiological reports have underlined an alarming increase in the prevalence of obesity and eating disorders, strongly indicating the necessity to counteract this trend in modern societies. According to the results from the 2003-2004 National Health and Nutrition Examination Survey (NHANES), in the United States alone more than 66% of adults are either overweight or obese. This figure represents a 10% increase in the prevalence rate from NHANES III (1988-94) and a 19 % increase from NHANES II (1976 -80) (Prevalence is the percentage of the population that falls into the designated category.)(1). It is also estimated that obese individuals spend approximately 36% more on health services and 77% more on medications than average-sized people costing the U.S. economy over 117 billion dollars per annum (1). Data from CDC's Behavioral Risk Factor Surveillance System (BRFSS) shows that, in USA in 1990, among states participating in the BRFSS, 10 states had a prevalence of obesity less than 10% and no states had prevalence equal to or greater than 15%. By 1998, no state had prevalence less than 10%, seven

states had a prevalence of obesity between 20-24%, and no state had prevalence equal to or greater than 25%. In 2006, only four states had a prevalence of obesity less than 20%. Twenty-two states had prevalence equal or greater than 25%; Two of these states (Mississippi and West Virginia) had a prevalence of obesity equal to or greater than 30%. (Figure 1)

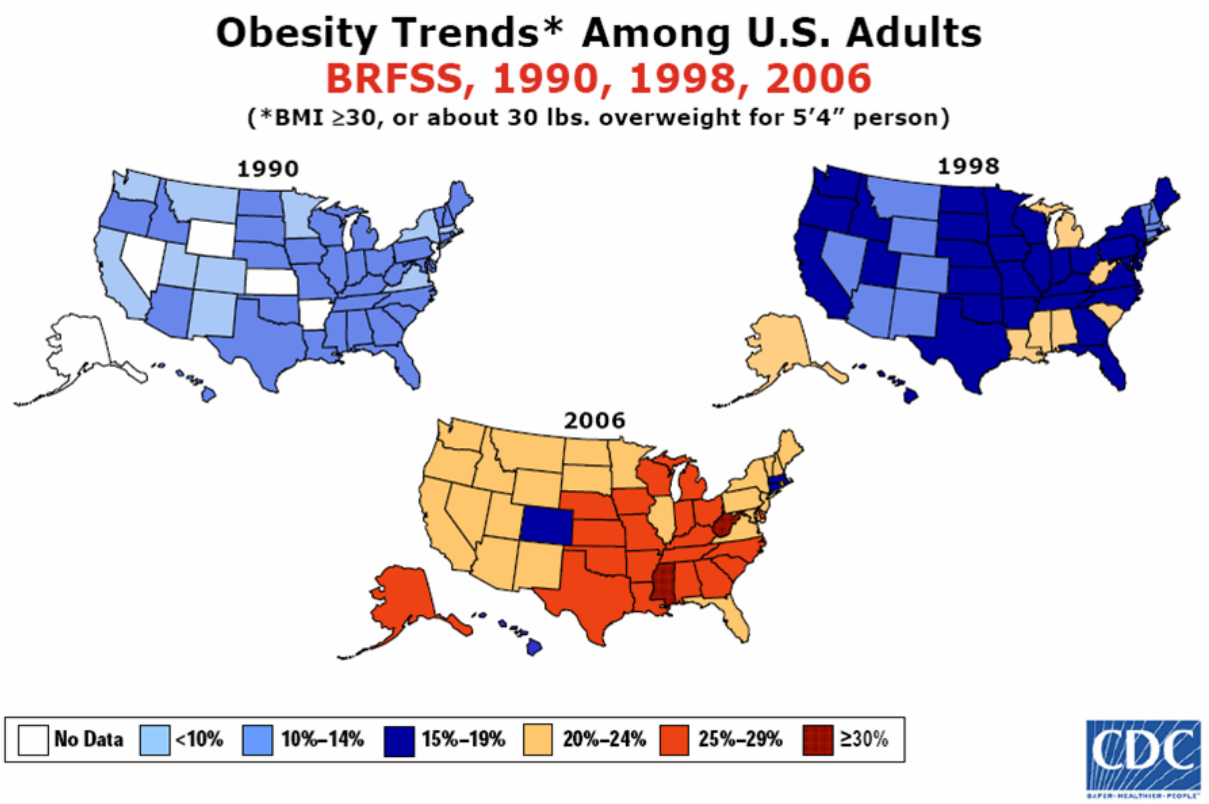


Figure1: BRFSS study of CDC done in 1990, 1998 and 2000 showing the prevalence of obesity in USA

Review of Literature

Obesity affects people of all ages and it results in complications affecting every system in the body. Serious health conditions such as Type II Diabetes Mellitus and cardiovascular disease are correlated with obesity (2, 3), and inappropriate regulation of consumption of highly palatable, fatty food is a major contributor to obesity (4).

Excessive food intake and sedentary lifestyle seem to be the major causes of obesity. It is believed that under pressure from frequent famines, thrifty genes were selected in certain populations of our ancestors that allowed the ingestion of large meals, relatively unopposed by satiety and guaranteed high fuel efficiency. In the modern world with easily available, energy dense foods, these survival genes have become a liability. Lifestyle changes attacking the real causes of obesity seem unattainable for many hence there is more emphasis on the pharmacological and surgical approaches to limit food intake or reduce fuel efficiency (5).

Regulation of food intake

Regulation of food intake is a very complex process and involves integration of the central and peripheral nervous system, the GI tract, and adipose tissue, as well as numerous hormonal and neurochemical signals. Feeding behavior is a functional output of distributed neural networks, with critical components located at every level of the central neuroaxis (6). Recent studies have focused on the identification of the molecular basis, the neuronal networks and metabolic pathways involved in the control of body weight and the regulation of food intake. It has been established that both central and peripheral signals contribute to the complicated circuitry that regulates feeding and energy homeostasis (7). Afferent sensory fibers are the primary neuroanatomic link between food-related events in the gut and the central neural

substrates that mediate the control of food intake (8, 9). These signals are detected by all senses, particularly smell, taste and visceral signals from the gut. They often involve learning and memory as well as reward and are initially processed mainly by cortico-limbic structures (5). The complex neural circuitry regulating feeding behavior involves the interplay of four classes of signals (5).

1. Signals conveying availability of potentially ingestible food in the environment. These signals are detected by all senses, particularly smell and taste. They often involve learning and memory as well as reward and are initially processed mainly by cortico-limbic structures (5). Studies from decerebrated rats have shown that short-term regulation of food intake and appetite control were intact to certain extent inferring that the brain stem can act independently without any coordination of higher brain regions (10).

2. Signals conveying availability of digestible food and absorbed nutrients in the alimentary canal. These signals are generated by the interaction of foods with mechano- and chemo-sensors all along the alimentary canal before, during and after absorption of nutrients and sensors in associated organs such as the liver and pancreas. These signals are detected either by primary afferent neurons of vagal and dorsal root origin or directly by the brain. They are processed initially in the nucleus of the solitary tract (NTS) and the area postrema in the caudal brainstem (5).

3. Signals conveying availability of circulating fuels and metabolites. Availability of glucose, for example, is detected at multiple peripheral and central locations (11). The arcuate nucleus has recently received a lot of attention for its ability to sense a variety of fuels and generate an integrated sense of metabolic status (5, 12).

4. Signals conveying availability of stored fuels such as fat in adipose tissue and glycogen in liver. Leptin, the best known of these signals, is detected at peripheral and central sites, including the arcuate nucleus (13). To understand control of food intake and regulation of energy balance, it is crucial to understand the rules and sites of integration of these many signals. Integration takes place in many brain areas, but studies suggest that hypothalamus and brainstem are the most important areas in regulation of food intake (5, 14).

Importance of hindbrain in control of food intake

The ability of any internal or external factor to modulate food intake ultimately depends on its ability to affect hindbrain neural circuits that control ingestive motor output. In this regard, anatomical, physiological, and behavioral data support the view that ingestive behavior outputs are modulated by neural signaling in the dorsal vagal complex (DVC), comprising the nucleus of the solitary tract (NTS), area postrema (AP), and dorsal motor nucleus of the vagus. The DVC receives relayed and direct synaptic input from olfactory, glossopharyngeal, facial, trigeminal, vagal, and spinal viscerosensory afferents that convey information about the chemical and mechanical properties of food. Medial portions of the DVC contain fenestrated capillaries that provide local parenchymal access to blood-borne factors (e.g., toxins, cytokines, hormones, osmolytes) that affect food intake (15). A number of neuroactive substances like leptin, neuropeptide Y (NPY), agouti-related peptide (AgRP), cholecystokinin, endocannabinoids, Pro-opiomelanocortin (POMC) related peptides such as Melanocyte Stimulating Hormone (α MSH) etc. have been suggested as being important to the control of food intake, and these are present in several nuclei of the brain (16).

Hypothalamic model

Earlier, the hypothalamus was considered as the primary focus of many peripheral signals and neural pathways that control energy homeostasis and body weight. In this hypothalamic centered model the interoceptors that detect blood-borne physiological signals and the integrative processes by which variations in physiological state exert their modulatory influences on the ingestive responses are localized to the hypothalamic nuclei. The 1994 discovery of leptin (17) revolutionized thinking about the neural systems that control energy balance and the physiology of white adipose tissue. Leptin's multimodal contributions to energy balance are attributed to its action on central nervous system (CNS) receptors (17). While the functional leptin receptor (ObRb) is expressed on neurons in various brain regions, attention has focused almost exclusively on the ObRb-bearing neurons of the arcuate nucleus of the hypothalamus. These neurons also express receptors for other peripheral signals, such as those for ghrelin and insulin, whose levels, like those of leptin, vary with energy status (18). The arcuate nucleus contains two anatomically and functionally distinct populations of neurons—the NPY/AgRP and the POMC/cocaine- and amphetamine-regulated transcript (CART) neurons. A series of important discoveries (19) showed that melanocortin receptor ligands [the agonist, α -melanocyte stimulating hormone α -MSH)], derived from POMC and the antagonist AgRP, made in the brain, affect feeding and energy expenditure through their actions on CNS melanocortin receptors (MC4-R and MC3-R). Mutations of the MC4-R result in hyperphagia and obesity in mice; similar effects on feeding and body weight are linked to mutations of the MC4-R in humans and may account for a significant percentage of pediatric obesity (20). Support for a critical role of arcuate nucleus neurons in energy balance also comes from studies showing that changes in energy status triggered by food deprivation or excessive caloric loading triggers alterations in the

gene expression of AgRP and NPY and of POMC and CART (21-23). These and a variety of other data support the hypothesis that arcuate nucleus neurons receive blood-borne and neural signals of relevance to the assessment of energy status and that the integration of these signals is reflected in the excitability of these cells. Through direct and multisynaptic projection pathways to caudal brainstem, spinal cord, and pituitary, the excitability of arcuate nucleus neurons is seen as directly controlling the three effector systems: behavioral, autonomic, neuroendocrine, whose activity maintains energy balance. This perspective, that a single site receives and integrates signals of relevance to energy balance control and issues commands to effectors, is described here as the arcuate model (24).

Distributive model

Emphasis has slowly moved away from anatomical concepts of ‘feeding’ and ‘satiety’ centers to centers outside the hypothalamus. In the *distributive model* feeding behavior is performed at more than one level of the neural axis (25). According to this model, several neurotransmitters and neuromodulators act in coordination in controlling feeding behavior and energy expenditure at different regions of brain like forebrain, arcuate nucleus of the hypothalamus as described above, hindbrain nuclei like the NTS and peripheral ganglia like the nodose ganglion. Recent studies indicate that the dorsomedial medulla in the hindbrain is very important in the control of food intake and bodyweight (26-44). This region plays a very important role because the caudal brainstem receives primary afferent information from the oral cavity pertaining to taste and also the primary afferent information from the gut (like, stomach, intestine, and liver) (45, 46).

The NTS of the dorsomedial medulla has been identified as the only brain region outside the arcuate nucleus that expressed the POMC gene. This suggests that the dorsomedial

medulla is an important center which acts as the convergence point for the integration and bidirectional flow of information, sensory afferent information towards the higher regions of the brain and the efferent motor information towards the periphery. Studies with decerebrate rats demonstrated that, in the absence of connections between forebrain and caudal brainstem that isolated caudal brainstem is sufficient to mediate many aspects of the short term feeding and energetic response to starvation that are attributed to hypothalamic signal processing (47), (24).

The NTS and the area postrema (AP) are two important regions of the dorsomedial medulla that are involved in the control of food intake. These regions are regarded as the chemoreceptor trigger zone for vomiting (AP) (26) and also these regions are suggested to play an important role in the inducing conditioned taste aversion (48). Several studies over the last couple of decades have shown that AP and the NTS immediately adjacent to the AP play an important role in the control of ingestive behavior and body weight regulation (26-28, 30-34, 36-38, 41, 42, 48-50). Rats with AP lesions are reported to consume increased quantities of highly palatable foods like sweetened condensed milk or glucose solution in short term tests (31, 34, 42, 51). This suggests that AP plays an important role in the modulation of short term food intake, especially for highly palatable foods. Behavioral studies with AP lesions and also the lesions of NTS adjacent to AP suggest that these regions play an important role in the selection of diet and body weight regulation (49). Animals with AP and NTS lesions showed a lesser decrease in body weight when compared with animals with only AP lesions. These animals also showed increased appetite for highly palatable foods (49, 51). The AP projects extensively to the adjacent NTS in the medulla and to the lateral parabrachial nucleus in the pons (41, 52) and these nuclei in turn project heavily to the forebrain including the hypothalamus (53, 54). Thus, it is possible that NTS is one of the important relay nuclei that serves as a convergence point,

receiving information from the periphery, which is then processed to the hypothalamus and other forebrain regions for integration with other signals resulting in the effective control of food intake, body weight and energy homeostasis.

Afferent vagal nerve fibers detect the chemical composition of food entering the intestine. Messages traveling in these nerves help to inform the brain of the type and quantity of nutrients being ingested. The brain then produces the sensation of “fullness” or “satiety,” which normally causes us to stop eating. Research on the control of food intake, as mentioned earlier has focused on mechanisms involving several neuroactive substances through which food intake is regulated. Cholecystokinin (CCK) and endocannabinoids are two of the neuroactive substances that are well established to play an important role in the regulation of food intake.

Role of CCK and cannabinoids in control food intake

Cholesystokinin (CCK)

Several peptides are released into circulation at the onset of eating or shortly thereafter. Some of these peptides like the intestinal peptides, CCK, pancreatic peptide, glucagon and amylin have been shown to play feedback roles in the control of the size of the meal that stimulates their release. CCK was the first gut peptide shown to play a role in the control of eating (55). CCK is released into extracellular space by I-cells of the small intestinal mucosa and it is suggested to be an important satiety signal from gut to brain (56). CCK is also very abundant in the brain. The only other neuropeptide which is more abundant in brain than CCK is neuropeptide Y (NPY). CCK levels are very high (> 4 ng CCK/mg protein) in cerebral cortex, caudate-putamen, hippocampus, and amygdala, while thalamus, hypothalamus and olfactory bulb are lower (1–2 ng/mg protein). The pons, medulla and spinal cord have even lower levels

(<1 ng/mg protein), while CCK is barely detectable in the cerebellum (57). CCK displays a high degree of tissue heterogeneity, and this appears to be species-dependent. CCK-8 is the predominant form of the peptide in the brain. In gut, larger forms like CCK 22, 33, 39, and 58 are more abundant than CCK 8. CCK acts by binding to the CCK receptors (CCKr) (58). Many of the effects of CCK, like stimulation of pancreatic exocrine secretion (59), inhibition of gastric emptying (60, 61), and satiation (62, 63), are mediated by activation of CCK-1 receptors on vagal afferent fibers (59, 61, 64-67).

CCK has been found to be colocalized with a number of "classical transmitters" and other neuropeptides with a distinct distribution. *In vitro* studies using brain slices have shown that the release of CCK can be elicited with potassium stimulation, veratridine, or an electrical field. Unlike the catecholamines, CCK requires a relatively strong stimulus for release, 40–60 mM potassium, compared with 25 mM for dopamine (68). Even in the presence of 60 mM potassium, CCK release can be modulated up or down by pharmacological agents (69-71). CCK increases excitatory amino acid release in the hippocampus (72). Most studies indicate that CCK increases gamma amino butyric acid release (73) while it decreases dopamine release (74).

Gibbs et al (75) showed that peripheral administration of CCK reduced short term food intake in rats in a dose dependent manner. Peripherally administered CCK inhibited feeding in most of the animals in which it was tested including humans (58). CCK release in the hypothalamus is increased after feeding in both rats and primates (76), and central administration of CCK causes satiety (77). The fat/fat mouse (which is obese, diabetic and sterile), whose primary genetic defect is loss of carboxypeptidase E (78), has about 20% of the whole brain CCK and about 60% of the duodenal CCK of its heterozygous littermates. This partial loss of CCK may contribute to the maturity-onset obesity of this mouse strain. CCK modulates food

intake by affecting the meal size and frequency (58). These effects of CCK are mediated by its endocrine actions in the gut and by its paracrine and neurocrine actions in the nervous system, especially the brain (58). The satiety actions of CCK are mediated by CCK1 receptor (58). The Otsuka Long-Evans Tokushima rat, which has no functional CCK1 receptors, is obese and does not respond to exogenous CCK, which supports the suggested physiological role of CCK (79). On the other hand, a transgenic mouse lacking the CCK1 receptor also does not respond to exogenous CCK, indicating that the CCK1 receptor has been eliminated. The exact mechanism by which CCK causes satiety is not clear, but inhibition of gastric emptying, production of hyperglycemia, and antagonism of the opiate feeding system and/or the endocannabinoid system are just a few possibilities (79, 80). It is likely that CCK is acting both centrally and peripherally in a complex fashion with other factors like cannabinoids, NPY, insulin, glucagon, serotonin, melanocortin and leptin in the regulation of feeding. As mentioned earlier, studies have established the role of NTS as one of the important relay nuclei that serves as a convergence point, receiving information from the periphery, which is then processed to the hypothalamus and other forebrain regions for integration with other signals resulting in the effective control of food intake, body weight and energy homeostasis. Cholecystokinin activates brainstem neurons in the nucleus tractus solitarius (NTS) and the area postrema (AP), primarily in the subregions where vagal sensory fibers terminate (80). CCK interacts with other neuroactive substances like leptin, insulin and endocannabinoids, suggesting a pivotal role in modulation of food intake (58).

Cannabinoids

The cannabis plants (*Cannabis sativa*) have been known since ancient times for their appetite-stimulating effects (81). Systematic observations of cannabis actions, by physicians in the 19th century, suggested potential clinical applications for treating the loss of appetite or body

weight (81). Recent studies on laboratory animals using the psychoactive compounds contained in cannabis such as Δ^9 -tetrahydrocannabinol (THC) and related cannabinoid molecules, indicate that cannabinoids induce appetitive behavior, confirming earlier observations. More recently, a physiological basis for the actions of plant-derived cannabinoids has been explained by the discovery of two cannabinoid receptors and their endogenous ligands. *N*-arachidonylethanolamine (anandamide), 2-arachidonoyl glycerol (2-AG), noladin ether, NADA, and virodhamine (82) are the endogenous cannabinoids identified. The cannabinoid CB1 receptor, is one of the most abundant G-protein-coupled receptors in the central nervous system, and is thought to be responsible for the majority of the 'central' actions of Δ^9 -tetrahydrocannabinol (Δ^9 -THC). CB2 receptors, are also G-protein coupled receptors of the $G_{i/o}$ family, but, these are poorly expressed in the brain under physiological conditions, and are found primarily in immune tissues and cells (83). CB1 and CB2 receptors share very little homology, thus pointing to a very early separation of the two encoding genes during evolution. The cannabinoid receptors, the endocannabinoids, and the proteins for their biosynthesis and degradation constitute the endocannabinoid system.

THC, or Δ^9 -tetrahydrocannabinol, the major active component of the marijuana plant (84), stimulates eating in people (85). Δ^9 -THC significantly increased intake, elevated hunger ratings, and enhanced food appreciation (86). Recent work using animal models has begun to shed light on the mechanisms by which THC acts to exert its actions on feeding. In particular, the discovery of cannabinoid receptors within the central nervous system and their endogenous ligands indicates that THC-induced effects reflect the modulation of key neural systems implicated in the normal control of appetite. Leptin, a key controller of hypothalamic anorexic and orexigenic mediators could significantly reduce the levels of anandamide and 2-AG in the rat

hypothalamus (87). Ghrelin, released into the bloodstream from the stomach during food deprivation in order to signal to the hypothalamus the need for energy intake, upregulates hypothalamic endocannabinoid levels (88). The fact that endocannabinoid levels are highest after food deprivation not only in the rat hypothalamus but also in the limbic forebrain, and possibly in the nucleus accumbens, which contributes to translating motivation to eat into action and this effect is blocked by using cannabinoid CB₁ receptor antagonist SR141716 (89). For the past few decades, THC has been shown to stimulate feeding in a variety of animal models, and this action has now been shown to be mediated by central-type CB₁ cannabinoid receptors, because THC-induced feeding is reversed by treatment with the selective CB₁ antagonist like rimonabant (SR141716). It has been demonstrated that CB₁ receptor antagonist SR 141716 [*N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methylpyrazole-3-carboxamide], preferentially suppresses the intake of foods high in carbohydrate content (90). SR 141716 administration in rats suppresses sucrose eating and drinking and reduces intake of a sweet milk dessert at doses that do not affect normal food or water intake (91, 92). The hyperphagic effect of THC in rats is highly potent, causing animals to overeat even when satiated (93). Studies have shown colocalization of CB₁ receptor with the appetite regulating hormones cocaine amphetamine regulated transcript (CART), melanin concentrating hormone (MCH), and corticotropin releasing hormone (CRH) in the paraventricular nucleus of the hypothalamus (94). Studies also show evidence for functional interactions between endocannabinoids and orexin A, an orexigenic peptide that is selectively expressed in the lateral hypothalamus and which has been linked to the stimulation of feeding (95). In addition to the hypothalamus, CB₁ receptors are also located in feeding-relevant hindbrain areas such as the dorsal motor nucleus of the vagus (DMV) and the NTS and may be subject to cannabinoid regulation (96) Earlier research from

our lab showed that the CB1 receptor is extensively distributed in the NTS and the administration of CB1 receptoreceptor agonist CP55, 940 into the fourth ventricle enhanced consumption of sweetened condensed milk with greater potency than when injected into the third ventricle (a thousand fold less dose was required), suggesting the importance of cannabinoids and hindbrain in the control of food intake, especially the highly palatable foods (97).

Vagal afferents and nodose ganglion

The vagal afferent neurons and the sympathetic (spinal) nerves are the major nerves, transmitting signals to the CNS from a variety of sensors in the gut in response to mechanical (distention, contraction) stimuli, various chemicals including nutrients in the gut lumen, neuro-hormonal stimuli such as gut hormones, neurotransmitters and neuromodulators as well as cytokines and inflammatory mediators produced by microbes in the gut (98). Although the chemical and colligative properties of food in the gut may directly activate vagal afferents, compelling evidence indicates that ingested foods trigger endocrine and paracrine secretions from the gastrointestinal mucosa, and that these neuroactive secretions in turn activate or modulate vagal afferent activity (99). CCK is secreted by intestinal I cells in response to fatty acids and protein in the intestinal lumen. The release of CCK reduces food intake by rapidly activating vagal afferent neurons (100). While other peptides secreted by the gastrointestinal tract may directly access the brain, vagal afferent activation appears to be the sole mechanism by which CCK from the intestine controls food intake (100, 101). Most of afferent vagal afferent neurons terminate on the NTS showing viscerotopic representation with fibers from esophagus and stomach ending at its rostral-lateral part, from stomach at caudal-medial part and from intestines at central and rostral parts of NTS (9). Connection by interneurons to the bodies of DMN completes the vago-vagal reflex pathways controlling various functions of the digestive

system giving it the autonomy to function on its own to regulate short term food intake. Some signals from the gut are transmitted onward toward higher neural centers *via* ascending tracts from the NTS up to the hypothalamus and its paraventricular nucleus (PVN), arcuate nucleus (ARC), central nucleus to amygdala (CAN) the bed nucleus of the stria terminalis (BNST) and the ventral thalamus to influence higher autonomic centers involved in appetitive behavior (9).

Interaction of CCK and cannabinoids

CCK reduces food intake by acting at CCK-1 receptors on vagal afferent neurons. Gastric and intestinal vagal afferents that express receptors for cholecystokinin (CCK) also express CB1 receptors. Effect of endogenous cannabinoids on appetite may be partially mediated by vagal afferent neurons (102). Expression of vagal CB receptors is increased by fasting and reduced by refeeding (103). CCK, which is released from the gut by food and believed to act as a satiety signal, also decreases CB1 receptor expression in vagal afferent neurons. It was also shown that the plasma CCK concentrations were low when expression of CB1 receptor increased with fasting and it was rapidly decreased with CCK administration or refeeding (103). CCK acts at low affinity states of CCK-1 receptors to trigger the entry of extracellular calcium into vagal afferent neurons (104). Recently ObRb mRNA has been reported in vagal afferent neurons, some of which also express CCK-1 receptor, suggesting that leptin, on its own or in cooperation with CCK, might activate vagal afferent neurons, and influence food intake via a vagal route (105). Unpublished data by Edwards et al., showed that the entry of extracellular calcium into vagal neurons was blocked by CB1 receptor agonist, CP55,940. Studies using recordings in solutions that contained only sodium or potassium revealed that CCK is capable of increasing a sodium dependent conductance or inhibiting a potassium dependent conductance (105, 106). These studies suggest that inhibition of calcium

channels or inhibition of active potassium conductance may be a likely mechanism mediating cannabinoid effects on CCK activity.

Phenotypes of neurons in NTS and nodose ganglion

The NTS receives dense terminations from cranial and visceral afferents, but little is known about which NTS neurons are involved or what mechanisms are responsible for the controlled regulation of both short term and long term feeding behavior, body weight and energy homeostasis. It has been demonstrated that only certain neurons are activated by CCK. It was shown that both catecholaminergic neurons and neurons expressing neuropeptide Y were activated by peripheral administration of CCK, while neurons expressing neurotensin failed to respond to CCK treatment (107). Glutamate, which is an excitatory neurotransmitter is released by the primary afferent fibers, and activates non-N-methyl-D-aspartate (non-NMDA) receptors on second-order neurons in the NTS. Both NMDA and non-NMDA receptors coexist on the same second-order NTS neurons and mediate primary visceral afferent transmission in the NTS (108). Intraperitoneal administration of cholecystokinin (CCK) to POMC-enhanced green fluorescent protein (EGFP) transgenic mice induced c-Fos gene expression in NTS POMC-EGFP neurons, which suggests that they are activated by vagal afferents stimulated by CCK (109). Several neurotransmitter substances and neurochemicals like glutamate (Glu), catecholamines, 5-HT, acetylcholine, numerous neuropeptides, such as substance P (SP), neurokinin A, vasoactive intestinal peptide, calcitonin gene-related peptide (CGRP), somatostatin (SS), and CCK and other neuroactive molecules (e.g. nitric oxide), have been identified in the neurons of the nodose ganglia (110). Vagal primary afferent neurons that play an important role in the mediation of 5-HT-dependent luminal stimuli, like hyperosmolarity and maltose, contain mainly Glu and SP (111).

Networks mediating feeding behaviors can be more thoroughly understood by phenotypic characterization of these neurons in light of the data regarding the patterns of CCK suppression by CP55,940.

This background information about the importance of hindbrain (especially NTS), and importance of CCK and cannabinoids in regulating/modulating feed intake, is the basis for my research.

Significance

It has been established that endogenous CCK participates in the short-term regulation of food intake (112, 113), thus, it is possible that the inhibition of this satiety signal would contribute to an increase intake of palatable, high caloric food. It has been shown that the administration of exogenous cannabinoids increases intake of highly palatable food (114). Fluorescent imaging studies with Fura 2 dye by Simasko et al. showed that CCK acts at the low-affinity site of the CCK-1 (CCK-A) receptor to trigger the entry of extracellular calcium into vagal afferent neurons (115). CB1 receptor agonist CP55,940 decreased or inhibited the entry of extracellular calcium into isolated vagal afferent neurons collected from nodose ganglion [Edwards et. al., unpublished data]. These studies suggest that cannabinoids suppress CCK's actions in the brain.

Such an interaction can be determined by the use of the immediate-early gene c-Fos. The c-Fos is a cellular proto-oncogene belonging to the immediate early gene family of transcription factors. Transcription of c-Fos is upregulated in response to many extracellular signals like growth factors. Expression of c-Fos in neuro-histochemical studies is used as an indirect marker of neuronal activity because c-Fos is often expressed when neurons fire action potentials (116). It

is established that expression of c-Fos is stimulated by intraperitoneal injection of cholecystokinin (80, 107, 117-119). Studies using camostat mesilate, a nonnutrient releaser of endogenous CCK decreased food intake and increased c-Fos like immunoreactivity in dorso-vagal complex and the myenteric plexus (120). In contrast, the cholecystokinin antagonist L364,718 has been demonstrated to decrease the number of cells expressing c-Fos protein in the NTS (117). So if exogenous cannabinoids antagonize the effects of CCK, the number of cells expressing c-Fos protein would be expected to decrease after subcutaneous injection of the cannabinoid agonist CP55,940. One plausible explanation for the interaction of CCK and cannabinoids can be that CCK when released in the gut or injected intraperitoneally, activates the CCK-1 receptors on the vagal afferents. The vagal afferents transmit this signal to the NTS neurons. CCK modulates the release of excitatory neurotransmitters like glutamate. Glutamate, which is an excitatory neurotransmitter is released by the primary afferent fibers, and activates non-N-methyl-D-aspartate (non-NMDA) receptors on second-order neurons in the NTS thereby activating the neurons which can be detected by the expression of c-Fos protein (Figure 5). Both NMDA and non-NMDA receptors coexist on the same second-order NTS neurons and mediate primary visceral afferent transmission in the NTS (108). The CB1 receptors are expressed presynaptically on the membranes of vagal afferent neurons, so when cannabinoid agonist CP 55,940 is injected ICV, it activates the CB1 receptor that inhibits the glutamate release thereby decreasing the activity of NTS neurons so the c-Fos activity in these neurons will be decreased or inhibited.

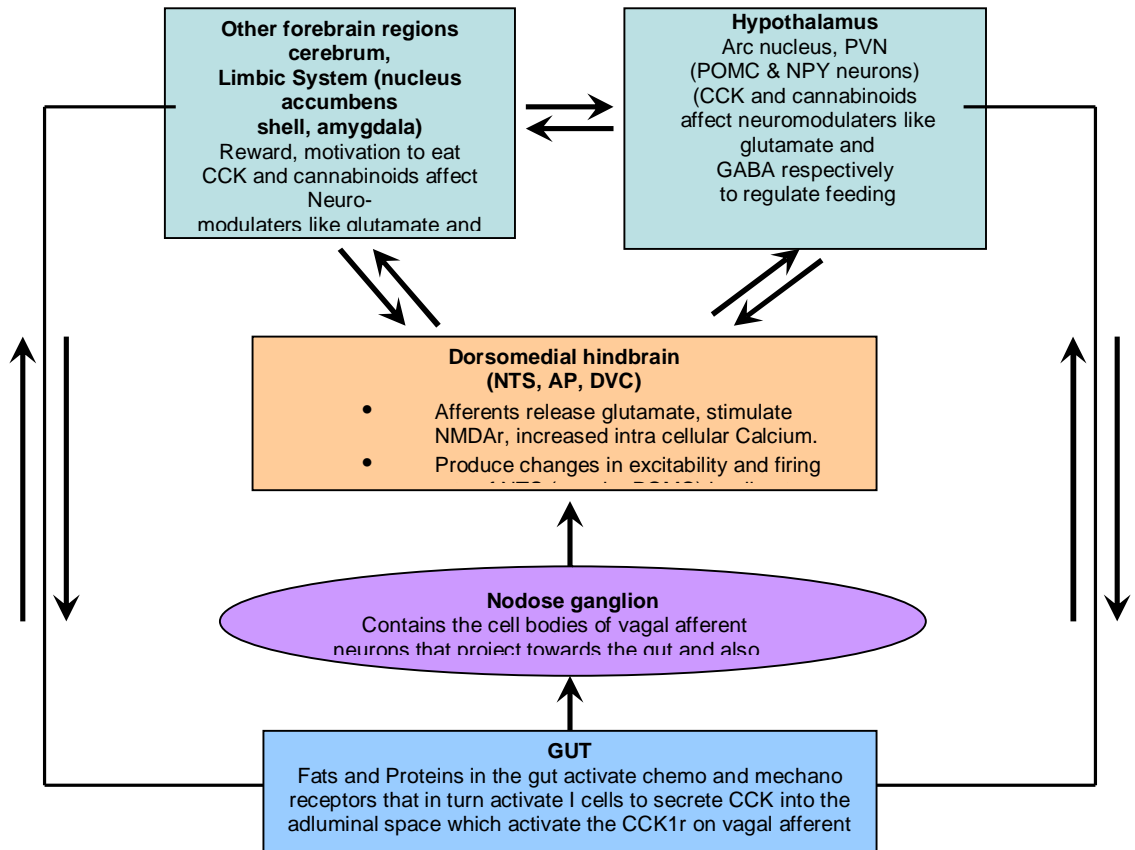


Figure 4: Central and peripheral regions involved in regulation of food intake showing areas of possible interactions of CCK and cannabinoids.

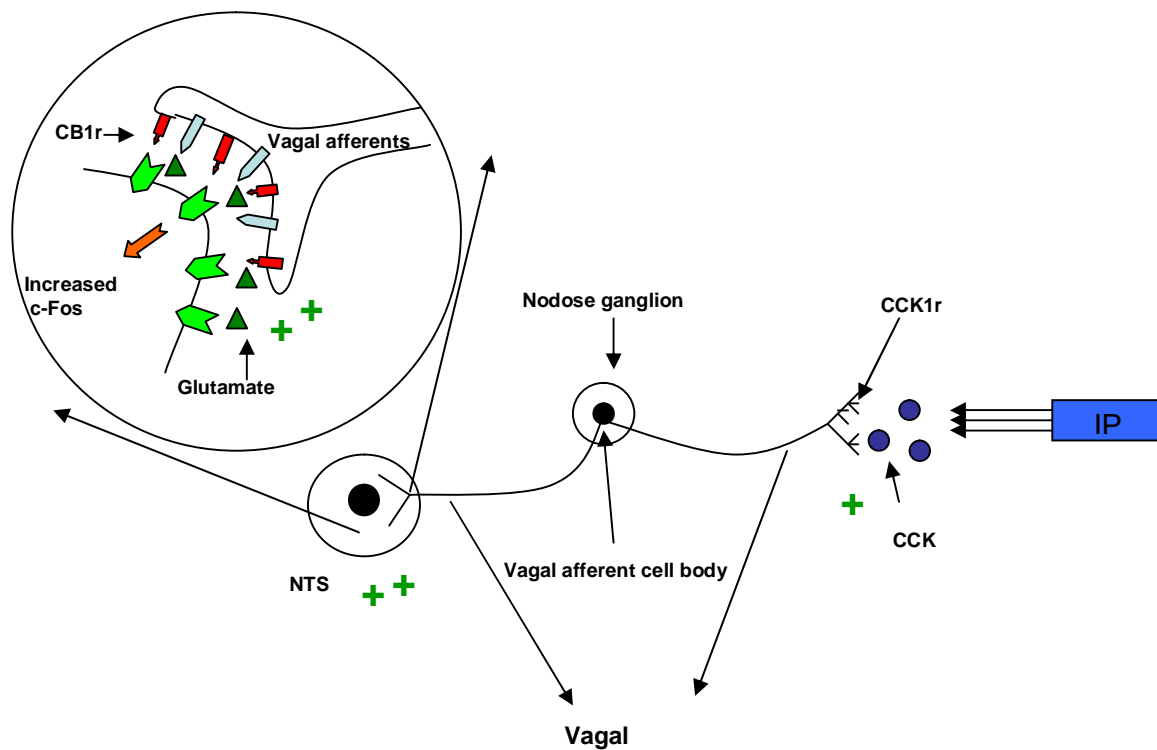


Figure 5: Depiction of the activation of c-Fos in NTS neurons by CCK. Injection of CCK (IP) stimulates vagal afferents by binding to the CCK1 receptors. The activation of vagal afferents by CCK causes the release of glutamate, an excitatory neurotransmitter that binds to non-NMDA receptors and activates NTS neurons which causes the expression of c-Fos protein. Cannabinoid agonist CP 55,940 binds to the CB1 receptors on the presynaptic membranes of vagal afferent neurons synapsing on the NTS neurons and inhibits the glutamate release thereby decreasing the activation of NTS neurons.

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Cannabinoid agonist CP 55,940 suppresses the neuronal activation by cholecystinin in the nucleus of solitary tract and area postrema¹

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Abstract

The nucleus of the solitary tract (NTS), and area postrema (AP) located in the dorso-medial brainstem are major regions of the hindbrain important in regulation of food intake. Cannabinoids increase the consumption of highly palatable foods. Earlier studies from our lab have shown that intracerebroventricular (ICV) administration of cannabinoid agonist, CP55,940 into the 4th ventricle increased the consumption of sweetened condensed milk. Cholecystokinin (CCK) is a well-studied satiety signal that plays an important role in the regulation of short-term food intake. Neurons in the NTS and AP are shown to express c-Fos protein following intraperitoneal (IP) injection of CCK. Our hypothesis is that cannabinoids decrease CCK activation of c-Fos in NTS and AP neurons. Cannulated rats were given CP55,940 (0.5µg/5µl/animal) via the 4th ventricle and were administered CCK (5µg/kgB.Wt, IP), 15 minutes after CP55,940 injection. Our results from this study showed that CP55,940 suppresses the c-Fos expression activated by CCK. The c-Fos counts in the CP/CCK treated rats, (NTS 69 ± 19 , AP 30 ± 6 ; mean \pm SEM) were significantly lower than the counts in the saline/CCK treated group (NTS 371 ± 94 ; AP 150 ± 47 ; mean \pm SEM) ($P < 0.05$). Results using the cannabinoid 1 receptor (CB1 receptor) antagonist SR 141716, CP55,940 and CCK showed that the inhibitory effect of CP55,940 on c-Fos activation of CCK was decreased using the SR141716. The c-Fos counts in the SR/CP/CCK treated rats were significantly higher than those from rats treated with saline/CP/CCK (104.3 ± 40.12 vs 24.29 ± 6.08).

This study confirms that CCK induced c-Fos like immunoreactivity in the NTS and AP neurons. Our study also showed that cannabinoid antagonist SR141716 decreased the inhibitory effect of CP55,940 on neuronal activation of CCK suggesting that this effect is mediated through the CB1 receptor.

Key Words: CCK, Cannabinoids, CB1 receptor antagonist, c-Fos, nucleus of solitary tract, area postrema.

Introduction

In both humans and animals, the gut is an important source of negative feedback controls of food intake (1, 2). When a substantial amount of food is present in the gut, it activates several neuroendocrine mechanisms that signal the brain to terminate the meal (3). Cholecystikinin (CCK), which is released in response to intracellular lipid or protein (4), is one of the most important mediators of gastrointestinal satiety. Further, CCK is also distributed widely throughout the brain and there are two types of CCK receptors present in the brain. CCK1 receptor is expressed at relatively low levels while the CCK2 receptor is densely distributed in the brain (5). In rats and humans, studies indicate that administration of selective antagonists of CCK1 receptor reduced the satiating effect of CCK and increased meal size. In addition, exogenous CCK-8, an octapeptide, decreased food intake through CCK1 receptors acting through an intact vagus nerve (6-8). Exogenous CCK-8 increased c-Fos expression in the dorsal vagal complex (9) and the myenteric plexus of the duodenum and jejunum through CCK1 receptors (10, 11).

Endocannabinoids, apart from the well-known psychological effects, cause an increase in appetite. Endocannabinoids, such as anandamide, are lipid-like neurotransmitter molecules activating the cannabinoid (CB1) receptors in the central nervous system (12). The fact that endocannabinoid levels are highest after food deprivation in the rat hypothalamus and in the limbic forebrain, and possibly in the nucleus accumbens, contributes to translating motivation to eat into action and this effect is blocked by using cannabinoid CB1 receptor antagonist

SR141716 [5-(4-bromophenyl)-1-(2,4-dichlorophenyl)-4-ethyl-*N*-(1-piperidinyl)-1*H*-pyrazole-3-carboxamide] (13). CB1 receptor, like CCKr, is found throughout the brain and is present at a high density in neocortex and hippocampus (14). Moreover, CB1 immunoreactivity in these brain areas, is found on CCK-expressing interneurons and later CB1 receptors were reported to be highly co-localized with cholecystinin and partially co-localized with calcium binding proteins (15).

CCK, which is released from the gut by food and believed to act as a satiety signal, also decreases CB₁ receptor expression in vagal afferent neurons. It was also shown that the plasma CCK concentrations were low when expression of CB1 receptor increased with fasting and it was rapidly decreased with CCK administration or re-feeding (16). Studies show that CCK acts at low affinity states of CCK1 receptor to trigger the entry of extracellular calcium into vagal afferent neurons (17). Recently leptin receptor b (ObRb) mRNA has been reported in vagal afferent neurons, some of which also express CCK-1 receptor, suggesting that leptin, on its own or in cooperation with CCK, might activate vagal afferent neurons, and influence food intake via a vagal route (18).

Cannabinoid agonists are shown to stimulate the activity of mesolimbic dopaminergic neurons and enhance brain stimulation-induced reward (19). The studies using the CB1 receptor antagonist SR141716 (20), suggested the importance of endogenous cannabinoid system in increasing the sensitivity of animal to stimulus by possibly modulating brain reward systems. Thus cannabinoid antagonist may reverse the effect of cannabinoid agonist by significantly increasing CCK-induced c-Fos activity in the neurons of NTS and AP.

The first objective of the current study was to first investigate the interaction of cannabinoid agonist CP55,940 and CCK in the NTS and AP. The second objective was to

demonstrate that suppression of CCK-induced c-Fos protein expression by cannabinoid agonist CP55,940 will be decreased by pretreatment of the rats with cannabinoid antagonist SR141716.

Materials and Methods

Animals

CD IGS rats were purchased from Charles River Laboratories, Wilmington, MA. Rats weighing 200–250g at the start of each experiment were housed in shoe box cages and were maintained on a 12:12-h light–dark cycle in a temperature-controlled environment (temperature 22±2 °C; humidity 50–60%). Standard laboratory rat chow pellets were obtained from Purina 5012, Purina Mills, St. Louis, MO and water were available ad libitum. All protocols were approved by the University of Georgia Institutional Animal Care and Use Committee.

Chemicals and Reagents

CCK8 peptide was purchased from New England Nuclear, Boston, MA and Cannabinoid agonist CP55,940 was from Tocris Cookson, Ellisville, MO. Cannabinoid antagonist SR141716 was obtained from National Institute on Drug Abuse, Rockville, Maryland. Primary antibodies for c-Fos and tyrosine hydroxylase were from Oncogene Science Inc, Uniondale, NY and Affinity Bioreagents, Golden, CO respectively. Cy3 antibodies were purchased from Molecular Probes, Eugene, OR and diaminobenzidine (DAB) kit was from Vector labs, Burlingame, CA.

Experimental Procedures

Surgery and cannula placement in 4th ventricle

A 22-gauge stainless steel cannula (Plastics One, Roanoke, VA) was implanted in the fourth cerebroventricle. Each rat was anesthetized with a cocktail of ketamine–acepromazine–xylazine (50:3.3:3.3 mg/kg im). The dorsum of the head and neck was clipped and vacuumed to remove loose hair. The anesthetized rat was placed in a stereotaxic apparatus with the neck

flexed. The surgical area was cleaned with chlorhexidine to disinfect the skin. An incision was made in the skin over the skull, and the periosteum was removed from the calvarium. Burr holes were drilled for the placement of two anchor screws and the cannula. The coordinates used for placement of cannula into the fourth ventricle were 2.0 mm rostral to the parietal–occipital suture on the midline and 7.5 mm ventral from dura, with the skull level between lambda and bregma (21). Dental acrylic was used to embed the screw heads and cannula, thereby fixing the cannula in place. When the acrylic was dry, a 30-gauge obturator was placed in the cannula. A 7- to 10-day postsurgical recovery period was allowed for body weights to stabilize prior to training the rats for intracerebroventricular injections.

Histological verification of cannula placement was performed in all rats after injecting methylene blue dye before euthanizing the animals, and any rat with improperly placed cannula was omitted from data analysis.

All drug trials were set up using a random design with CP55,940 and SR141716 depending on the experiment. Rats were intracerebroventricularly injected with CP55,940/SR141716 or vehicle using a Gilmont micrometer syringe fitted to an injector that extended 1 mm beyond the cannula tip. 50% DMSO and 50% saline was used as a diluent for CP55,940/SR141716. However, we have previously found that rats intracerebroventricularly (ICV) injected with a solution containing 50% DMSO and 50% saline did not significantly alter the intake of sweetened condensed milk compared to rats injected with saline alone (data not shown). For this reason, control animals in these studies received saline alone.

Experiment 1: Effect of cannabinoid agonist CP55,940 on neuronal activation of CCK in NTS and AP.

It was reported from our lab earlier that central CP55,940 increases the consumption of palatable food (22). The aim of Experiment 1 was to demonstrate by immunohistochemistry that CP55,940 was effective in significantly suppressing the c-Fos activity in the neurons of NTS and AP by CCK. In this study, 25 male rats were used. Control rats received either saline/saline (IP/ICV) or saline/5 µg/kg of CCK (IP/IP). The treatment group rats received 0.5 µg of CP 55,940 into the 4th ventricle and 5 µg/kg of CCK (IP).

Experiment 2: Effect of cannabinoid agonist CP55,940 on CCK-induced c-Fos activity in NTS and AP upon pretreatment of the rats with cannabinoid antagonist SR 141716.

Our studies with the central administration of CP55,940 showed by immunohistochemistry that the neuronal activity of the neurons in NTS and AP increased by CCK was suppressed significantly by the central administration of CP55,940. We believe that this activity was facilitated by the CB1 receptor, so to find out that this interaction was indeed facilitated by CB1 receptor, we used a cannabinoid antagonist SR141716, to see if the suppression of c-Fos activity by CP55,940 can be blocked/reversed by the administration of SR141716, a CB1 receptor antagonist. Male rats (n=20) were used in this study as well. Drug administration was similar to the first experiment except that the animals receiving CP55 940 and CCK were pretreated with 6µg SR141716 (4th ventricle).

Immunohistochemistry

In both the studies, 2 h following treatment, rats were euthanized with an overdose of pentobarbital based euthanasia solution and the animals were injected with 10 µl of methylene blue ICV for the verification of cannula placement later. The rats were then transcardially

perfused with heparinized 0.9% saline (0.1 ml heparin/100 ml saline) and 200 - 300 ml of ice cold, phosphate buffered 4 % paraformaldehyde. After perfusion the brains were removed and postfixed in paraformaldehyde with 10% sucrose for 3 h. The brains were then transferred to 30% sucrose solution for 48 h for cryoprotection after which they were wrapped in aluminum foil and stored at -80°C for immunohistochemistry.

The brains were cut into 20 µm transverse sections using a cryostat. Sections were first incubated in 0.3 % hydrogen peroxide for 30 min. The sections were then pre-incubated in 0.3 % triton-X 100 (PBS-TX) with 4% normal goat serum (NGS) for 2 h at 4°C. The sections were then incubated in primary antibody (1:40,000 rabbit anti-c-Fos in PBS-TX with 1 % NGS) for 48 h, followed by incubation with the secondary antibody (1:200 biotinylated goat anti-rabbit) for 1 h. Sections were then incubated for 1 h in avidin-biotin complex solution (Standard Elite VECTASTAIN ABC kit, Vector Laboratories, Burlingame, CA). DAB solution was added for 5-min incubation and the reaction was stopped with a PBS wash. Sections were thoroughly washed between each incubation period.

Sections were mounted on gelatin coated slides and air dried. Once dry the sections were washed in a series of ethanol solutions for dehydration and xylene for delipidation. The sections were then coverslipped using Permount histological mounting medium (Fisher Scientific, Fair Lawn, NJ). Sections were viewed using a Nikon light microscope and the images were taken with a Ziess Axioplan 2 imaging microscope using SPOT Basic software and quantified using Image-Pro Plus software (Media Cybernetics, Inc, Bethesda, MD).

A stereotaxic rat brain atlas was used to determine the location of the NTS in each section of tissue (21). A region of interest was drawn around the NTS, and all activated neurons in the NTS region of interest were counted. Neurons were considered immunopositive by the intensity

of staining and size. An average of 10 representative sections per animal was chosen, corresponding to different levels of the NTS and AP. The counts used in statistical analysis represent the average number of NTS and AP neurons showing positive immunoreactivity for c-Fos

Statistical analysis

Statistical analysis was performed using a one-way ANOVA by counting the number of cells in the NTS and AP showing c-Fos protein. A value of $P < .05$ was used to establish statistically significant differences.

Results

CP55,940 suppressed neuronal activation by CCK in NTS and AP

The results of Experiment 1 showed that CP55,940 injected into the 4th ventricle of rats significantly decreased the expression of c-Fos protein induced by IP administration of CCK. Administration of CCK alone increased neuronal activity as indicated by the number of neurons expressing c-Fos protein in both NTS and AP (NTS, 371 ± 94 ; AP 150 ± 47 ; mean \pm SEM) (Figure 1A & B). About 10 sections were used for counting neurons per each animal and the average counts per section \pm SEM were plotted in the figures. The effect of CCK was decreased significantly by the ICV administration of CP 55,940 (NTS, 69 ± 19 ; AP 30 ± 6 ; mean \pm SEM) (Figure 1A & B). The animals which received saline (IP/ICV) showed minimal neuronal activation (NTS and AP are 21 ± 3 ; 9 ± 1 ; and AP 7 ± 2 , respectively; mean \pm SEM) (Figure 1A & B). Representative hind brain images of c-Fos immunoreactivity in neurons of NTS and AP are demonstrated in Figure (2A, B & C).

CP55,940 suppression of CCK-induced c-Fos immunoreactivity is inhibited by cannabinoid antagonist SR141716

In the second experiment, we showed that SR141716 reversed the effect of CP55,940. This reversal was evident from an increase in the c-Fos immunoreactivity in the NTS of animals pretreated with AP of animals SR 141716 before injecting CP55,940 (ICV) and CCK (IP) (104.3 ± 40.12 vs 24.29 ± 6.08). Interestingly, the animals that were injected with SR 141716 (ICV), saline (ICV) and CCK (IP) showed higher c-Fos protein expression than the group which received all the three drugs (218.5 ± 85.5 vs 104.3 ± 40.12). As expected, the animals that received either SR 141716 (22.5 ± 18.5) or CP 55,940 (21.5 ± 10.5) alone showed minimal neuronal activity. Likewise, the results of neuronal activity in AP were very much similar to that of NTS. The animals that were injected with SR141716 (ICV), saline (ICV) and CCK (IP) showed higher c-Fos protein expression than the group which received all the three drugs (314.5 ± 6.5 vs 98.29 ± 50.05). Similarly, the animals that received either SR 141716 (14.5 ± 8.5) or CP 55,940 (12 ± 1) alone showed minimal neuronal activity.

Discussion

In the current study the IP injection of CCK induced c-Fos immunoreactivity in the neurons of NTS and AP as previously described (23, 24). Further, we have shown that the cannabinoid agonist, CP55,940, injected into the fourth ventricle significantly suppressed the c-Fos immunoreactivity induced by IP administration of CCK.

CCK is released adluminally by the interstitial cells of the gastrointestinal mucosa and it is an important satiety signal from gut to brain (25). CCK was also shown to act as neurotransmitter or neuromodulator to produce satiety when injected into NTS and medial-basal hypothalamus (26). The satiety actions of CCK are mediated by CCK1 receptor (27). The

Otsuka Long-Evans Tokushima rat, which has no CCK1 receptors, is obese and does not respond to exogenous CCK, which supports the suggested physiological role of CCK in controlling appetite (28). Furthermore, studies using camostat mesilate; a nonnutrient releaser of endogenous CCK decreased food intake and increased c-Fos like immunoreactivity in dorsovaginal complex and the myenteric plexus (29). Our results on the effects of CCK on c-Fos expression are in agreement with earlier studies where CCK-8 showed c-Fos like immunoreactivity in the cells of all the four subnuclei of NTS (30, 31). The expression of c-Fos protein in the neurons of NTS and AP in response to CCK has been shown in a variety of studies (23, 24). The stimuli associated with neuronal excitation elicit a transient induction of c-Fos mRNA, followed by c-Fos protein synthesis. The c-Fos is a cellular proto-oncogene belonging to the immediate early gene family of transcription factors. Transcription of c-Fos is upregulated in response to many extracellular signals like growth factors. Expression of c-Fos in immunohistochemical studies of neurons is used as an indirect marker of neuronal activity because c-Fos is often expressed when neurons fire action potentials. The accumulation of c-Fos mRNA reaches a peak at 45 min followed by subsequent protein expression (32).

Recent *in vivo* studies using cannabinoid molecules indicate that cannabinoids induce appetitive behavior (33). Miller et al in 2004 reported that administration of cannabinoid agonist CP55,940 into the fourth ventricle acts locally in the hindbrain to influence feeding behavior in rats (22). Further, the effective dose of CP55,940 was about 1000-fold lower when injected into the fourth ventricle compared to injection into the lateral ventricle of rats (22). The cannabinoid CB₁ receptor, is one of the most abundant G-protein-coupled receptors in the central nervous system, and is thought to be responsible for the majority of the 'central' actions of delta 9-tetrahydrocannabinol. Neurons located in the subpostrema area of the NTS were sensitive to

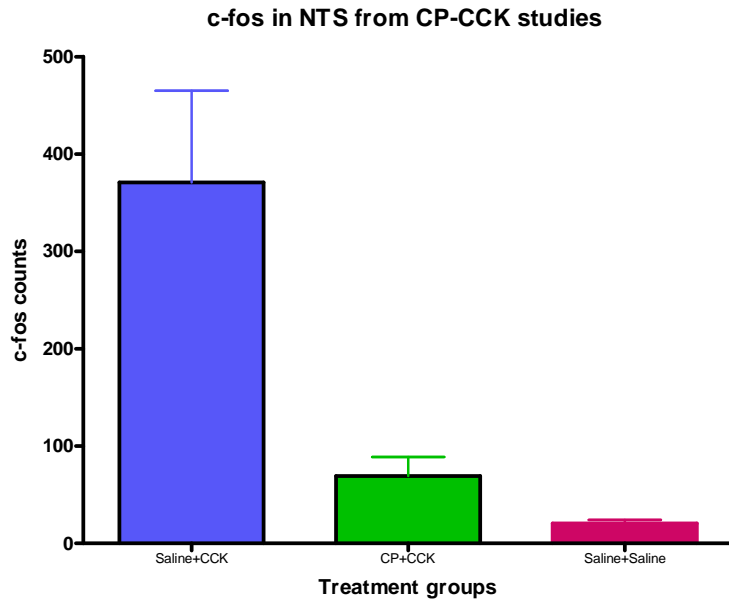
delta 9- tetrahydrocannabinol when applied to rat brain slices (34, 35) also indicating the localization of cannabinoid receptors in that area. In our studies, we have shown the CP55,940 injected into the fourth ventricle significantly suppressed the c-Fos immunoreactivity in NTS and AP neurons induced by IP injection of CCK. Studies investigating the extracellular recordings in rat brain slices showed that the spontaneous firing rate of 54.8 % of neurons was significantly changed after applications of delta 9- tetrahydrocannabinol. These sensitive neurons were depressed by clonidine, an α_2 agonist, and therefore are adrenergic or noradrenergic (34). Rinaman et al., showed that perIPheral administration of CCK activates catecholaminergic neurons with in caudal medulla (36). Therefore it is possible that this population of catecholaminergic neurons in the NTS that showed c-Fos like immunoreactivity induced by CCK (36) is also the same population of neurons sensitive to delta 9- tetrahydrocannabinol (34) and are playing an important role in the interaction of CCK and cannabinoids to regulate short term food intake. Glutamate, which is an excitatory neurotransmitter is released by the primary afferent fibers, and activates non-N-methyl-D-aspartate (non-NMDA) receptors on second-order neurons in the NTS (28). It is possible that CCK which activates the vagal afferent neurons releases glutamate that activates the neurons of the NTS. In addition, we have also demonstrated that CB1 receptor antagonist SR141716 reversed the inhibitory effect of CP55,940 on CCK induced c-Fos immunoreactivity. Our study with the CB1 receptor antagonist SR141716 shows that the inhibition of CCK activation of NTS neurons can be decreased or reversed to some extent. CP55,940 is a nonspecific cannabinoid agonist. It can bind to both CB1 and CB2 receptors. The cannabinoid antagonist SR141716 is CB1 receptor specific so the reversal of CP55,940 effect by SR141716 suggest that this interaction is mediated through CB1 receptor. CCK was shown to decrease CB₁ receptor expression in vagal afferent neurons (16).

The effects of delta 9- tetrahydrocannabinol on stimulating feeding in a variety of animal models has been shown to be mediated by central-type CB1 cannabinoid receptors, as delta 9- tetrahydrocannabinol-induced feeding is reversed by treatment with the selective CB₁ antagonist like SR141716 (37). CCK increased the frequency of spontaneous and miniature excitatory postsynaptic currents in 43% of the central NTS neurons via a presynaptic mechanism, using whole cell patch-clamp techniques (31). Unpublished data by Edwards et. al., showed that the entry of extracellular calcium into vagal neurons was blocked by CB1 receptor agonist, CP55,940. Moreover, studies using recordings in solutions that contained only sodium or potassium revealed that CCK is capable of increasing a sodium dependent conductance or inhibiting a potassium dependent conductance (38, 39). These studies suggest that inhibition of calcium channels or inhibition of active potassium conductance may be a likely mechanism mediating the inhibitory effects of cannabinoid on CCK activity.

In conclusion, our immunohistochemical studies showed that CP55,940 injected in the fourth ventricle significantly suppressed the c-Fos immunoreactivity induced by CCK. This further confirms the behavioral data published from our lab by Miller et al., that cannabinoids injected into the 4th ventricle have a greater influence on feeding, acting locally in the NTS and AP surrounding the fourth ventricle (22). Anatomical and behavioral data suggest the dorsal vagal complex as a likely site for hindbrain actions of cannabinoids to influence food intake. Our data strengthens this argument that the neurons in NTS and AP play an important role in the regulation of feed intake and that the interaction of cholecystokinin and cannabinoids is an important mechanism facilitating the regulation of feed intake in hind brain. Future studies are required to identify the subpopulations of the NTS neurons involved in the cannabinoids and cholecystokinin interaction.

Figures:

A.



B.

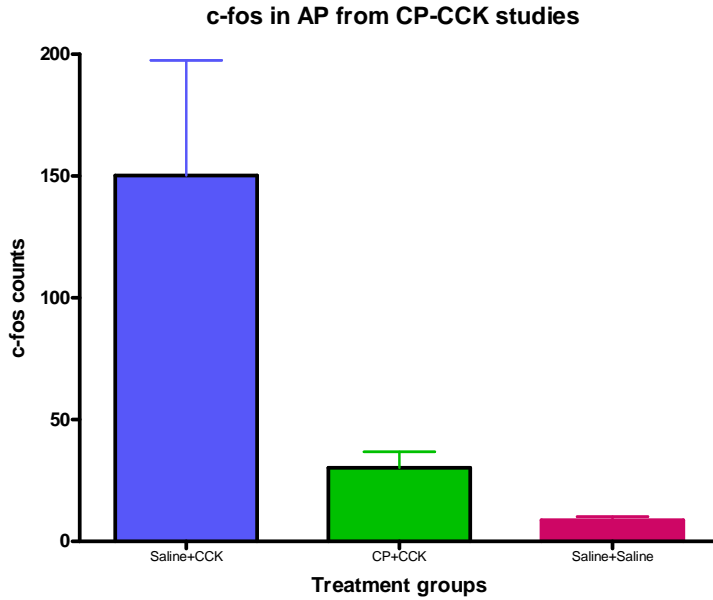
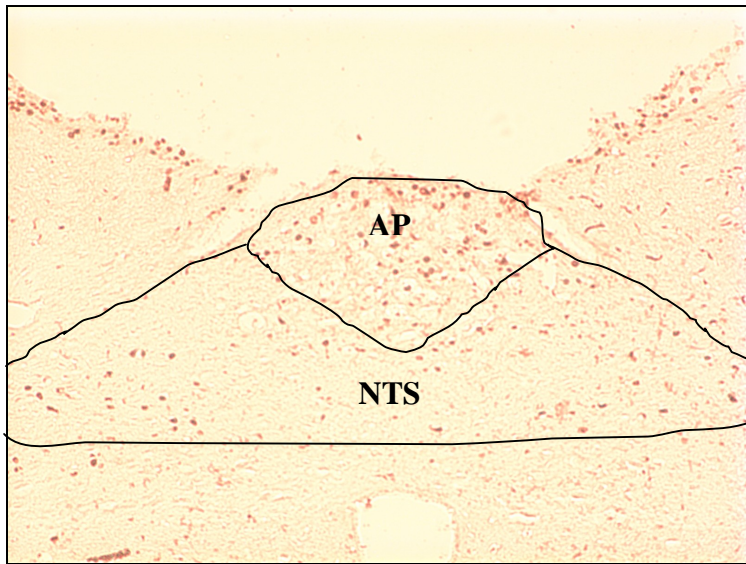
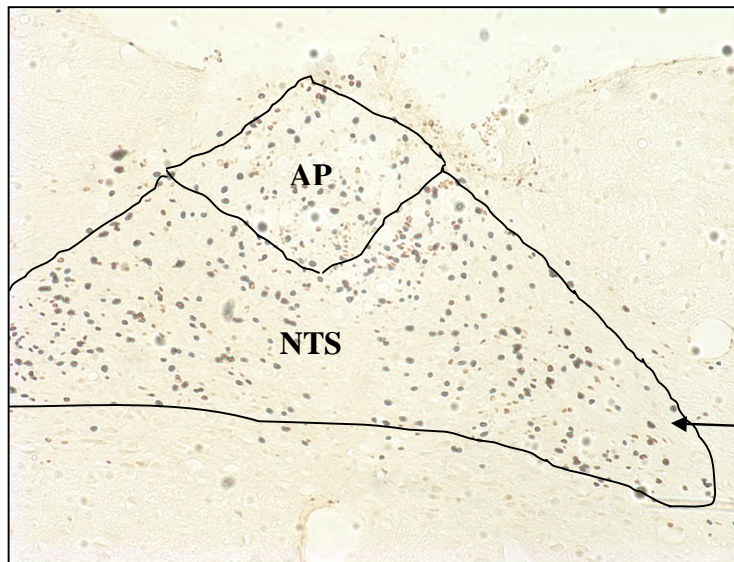


Figure 1. Number of c-Fos positive neurons (mean±SE) in NTS (A) and AP (B) of rats administered with CCK (IP)/CP 55,940 (ICV), saline (IP)/CP 55,940 (ICV) and saline (IP)/saline (ICV).

A.



B.



nuclei stained for c-Fos immunoreactivity

C.

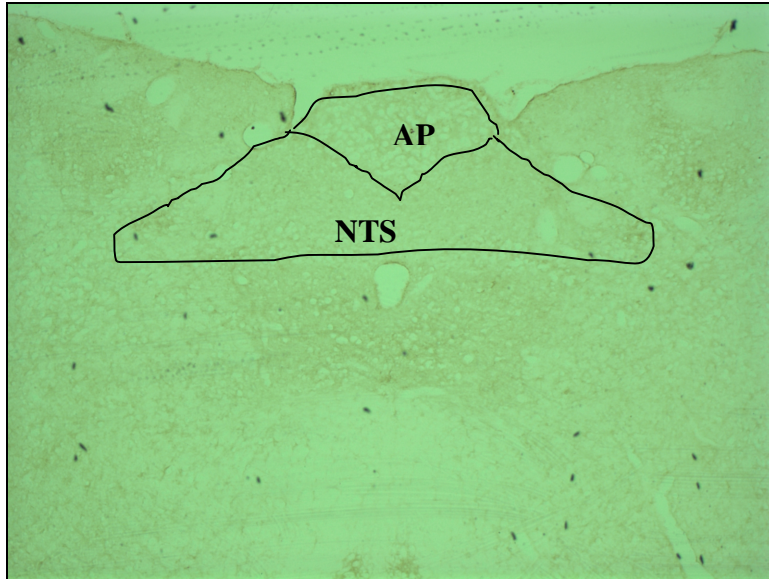
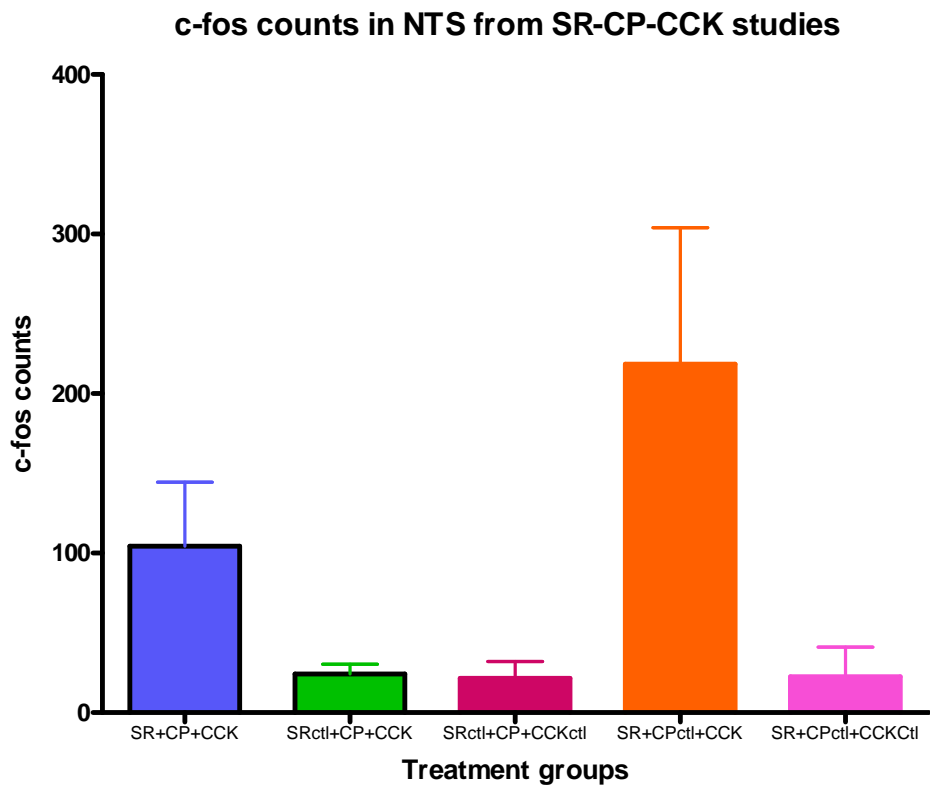


Figure 2. Photographs depicting c-Fos protein in the NTS and AP of rats (n=25) detected and counted using immunohistochemistry and image analysis. Sections of the dorsomedial region of hind brain illustrating the areas of NTS and AP where c-Fos positive neurons were counted. A. CCK (IP) + CP 55,940 (ICV), B. saline (ICV) + CCK (IP) and C. saline (IP) + saline (ICV).

A.



B.

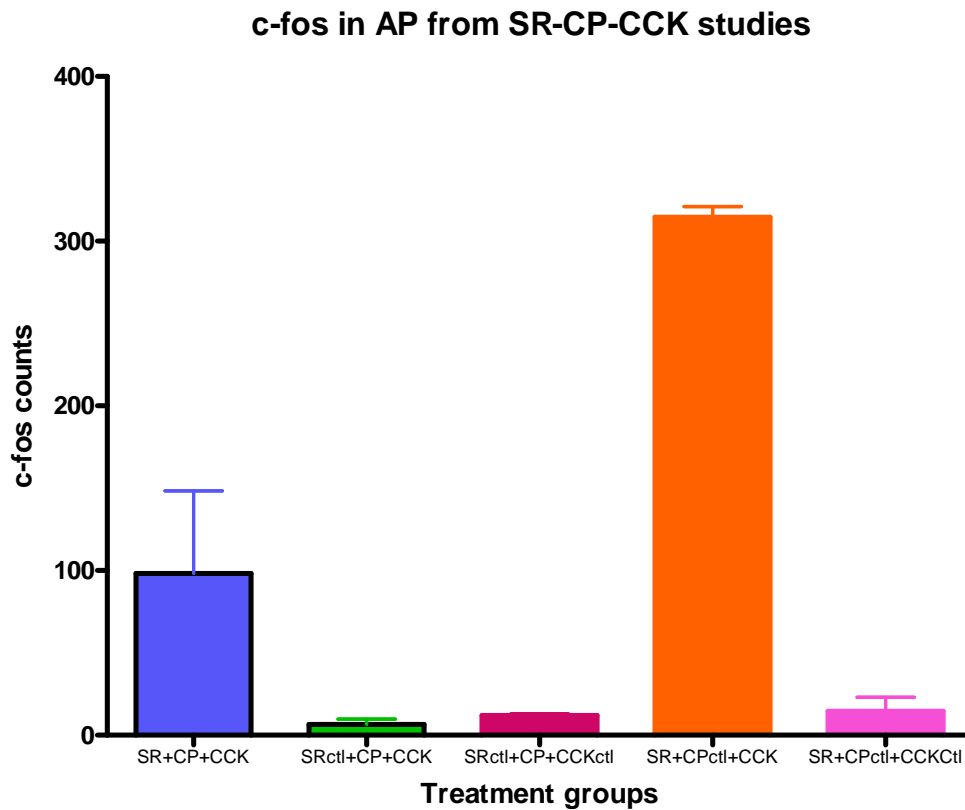
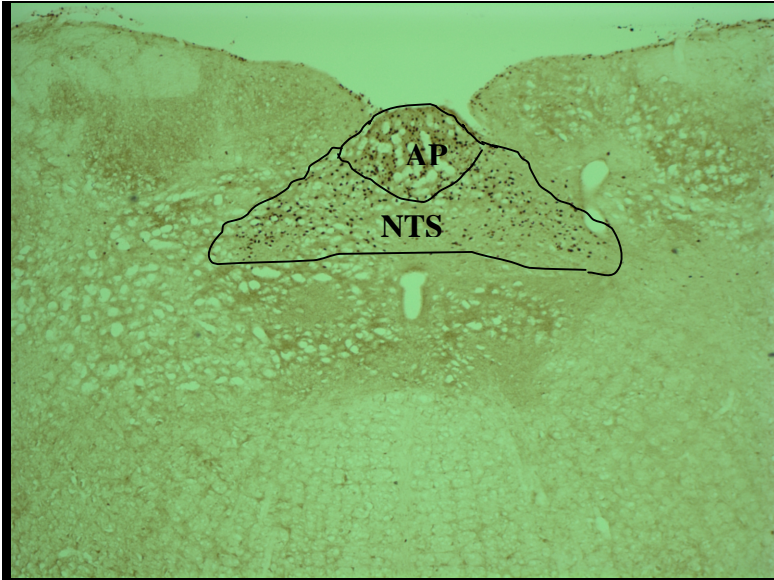
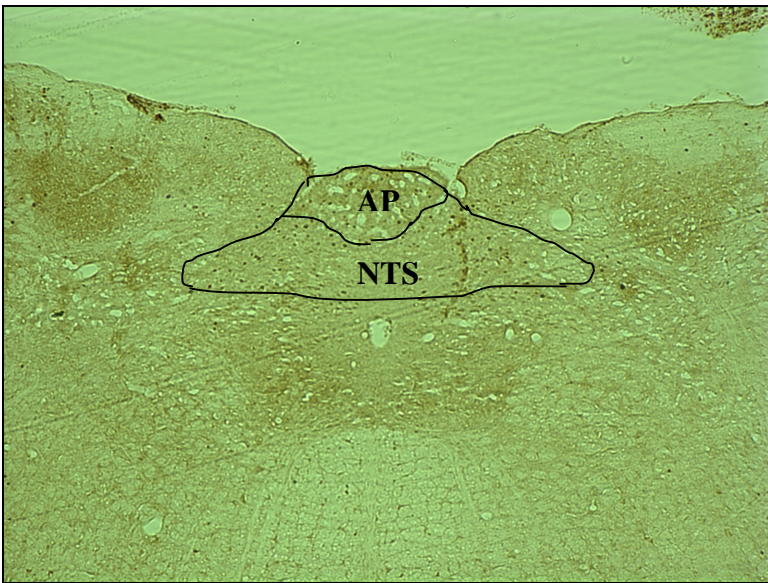


Figure3. Number of c-Fos positive neurons (mean \pm SE) in NTS (A) and AP (B) of rats administered with SR141716 (ICV) + CP 55,940 (ICV) + CCK (IP), B. saline (ICV) + CP 55,940 (ICV) + CCK (IP) and C. SR141716 (ICV)+ saline (ICV) + saline (IP). D. Saline (ICV)+ CP55,940(ICV) + saline (IP).

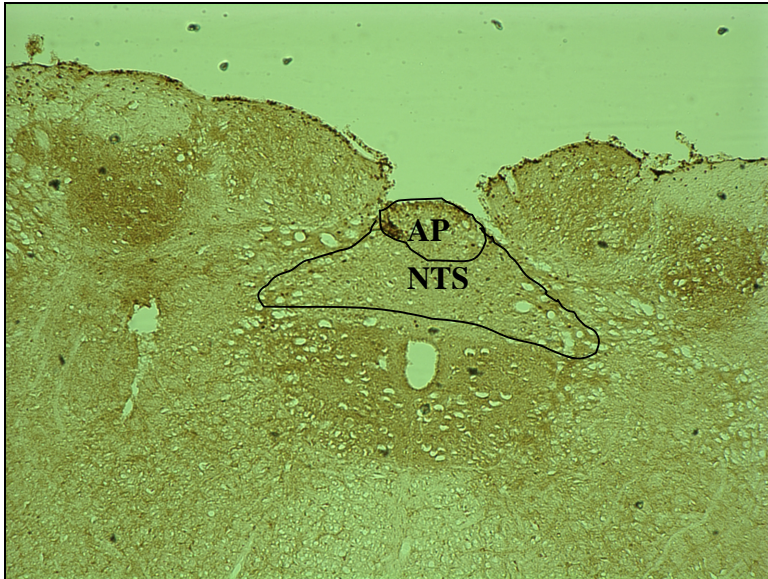
A.



B.



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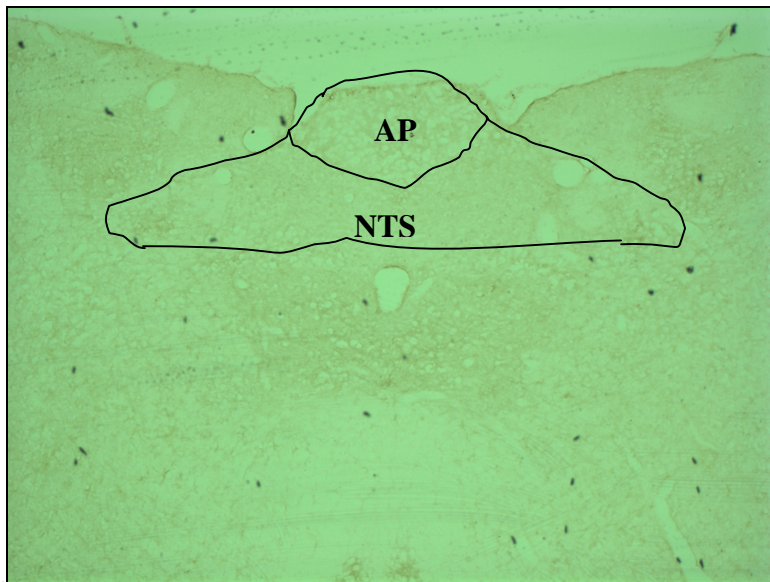


Figure 4. Photographs depicting c-Fos protein in the NTS and AP of rats (n=20) detected and counted using immunohistochemistry and image analysis. Sections of the dorsomedial region of hindbrain illustrating the areas of NTS and AP where c-Fos positive neurons were counted. A. SR141716 (ICV) + CP 55,940 (ICV) + CCK (IP), B. saline (ICV) + CP 55,940 (ICV) + CCK (IP) and C. SR141716 (ICV)+ saline (ICV) + saline (IP). D. Saline (ICV)+ CP55,940(ICV) + saline (IP).

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Catecholaminergic neurons of NTS activated by cholecystokin (CCK) are not involved in the inhibition of CCK activity by cannabinoid agonist CP55,940²

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Abstract

Cannabinoids have been shown to influence food intake, and until recently, the neural pathways mediating these effects have not been clearly understood. The nucleus of the solitary tract (NTS), and area postrema (AP) located in the dorsomedial brainstem are major regions of the hindbrain important in regulation of food intake. Earlier studies from our lab have shown that intracerebroventricular (ICV) administration of cannabinoid agonist, CP55,940 into the 4th ventricle increased the consumption of sweetened condensed milk. The c-Fos immunoreactivity studies from Chapter 1 show that cannabinoid agonist CP55,940 significantly suppressed the neuronal activation of CCK in NTS neurons. Cholecystokinin (CCK) is a well-studied satiety signal that plays an important role in the regulation of short-term food intake. Neurons in the NTS and AP are shown to express c-Fos protein following intraperitoneal (IP) injection of CCK. Our hypothesis for this study is that the effect of cannabinoids on CCK activation of c-Fos in NTS and AP neurons involves catecholaminergic neurons. The hindbrain tissues used in the first study were also used for the double labeling using c-Fos and TH antibodies. Our results from this study showed that CP55,940 suppresses the c-Fos expression activated by CCK. The c-Fos activity was found in TH positive cells but there was no statistically significant difference in the number of TH positive neurons showing the c-Fos immunoreactivity between the two treatment groups. The number of TH positive neurons showing c-Fos immunoreactivity in CP/CCK treated rats, (NTS 39 ± 6 ; mean \pm SEM) were not significantly different than the counts in the saline/CCK treated rats (NTS 42 ± 9 ; mean \pm SEM) ($P < 0.05$). Similarly the counts of TH positive neurons showing c-Fos immunoreactivity in the NTS of SR/CP/CCK treated rats (13 ± 1) were not significantly different from the rats treated with saline/CP/CCK (7 ± 1), SR/saline/CCK (13 ± 1), saline/saline CCK (15 ± 4). This study further confirms that CCK induced c-Fos like

immunoreactivity in the catecholaminergic neurons of the NTS. There is no statistically significant difference between the counts of c-Fos positive catecholaminergic neurons suggesting that the interaction of CCK and CP55,940 seems not to involve the catecholaminergic neurons in NTS.

Key words: CCK, Cannabinoids, CB1 receptor antagonist, c-Fos, nucleus of solitary tract, tyrosine hydroxylase

Introduction

Endocannabinoids are endogenous products, synthesized via a multi-enzymatic cascade from phospholipids. Upon demand, neurons produce endocannabinoids intracellularly, and when released, these endocannabinoids activate CB1 receptors (1). CB1 is found throughout the brain and is present at a high density in neocortex and hippocampus (2). In these brain areas, CB1 immunoreactivity is found on large cholecystinin (CCK)-expressing interneurons(3). Several reports have shown immunohistochemical staining for CB1 receptors on fibers within the nucleus tractus solitarii (NTS) (4, 5) indicating that the components necessary for endocannabinoid modulation of neurotransmission are present in the NTS. CB1 widely mediates endocannabinoid effects on glutamatergic and GABAergic transmission to modulate cortical networks (3).

Signaling of cellular metabolic insufficiency involves the hindbrain, area postrema (AP) and ventromedial hypothalamic nucleus (VMH) (6). Catecholaminergic (CA) neurons in the caudal hindbrain NTS/AP complex participate in the origin and/or relay of stimuli that signal metabolic deficiency to the brain (7). Further, substance P-like immunoreactive neurons in the NTS of the rat send their axons to the nucleus accumbens (ACB) (8) and many neurons with

tyrosine hydroxylase (TH), neurotensin (NT) or CCK immunoreactivities have been found in the rat NTS (9).

It has been demonstrated that only certain neuronal subpopulations are activated by CCK. It was shown that both catecholaminergic neurons and neurons expressing neuropeptide Y were activated by peripheral administration of CCK, while neurons expressing neurotensin failed to respond to CCK treatment (10). Moreover, intraperitoneal administration of CCK to POMC-enhanced green fluorescent protein (EGFP) transgenic mice induced c-Fos gene expression in NTS POMC-EGFP neurons, suggesting that they are activated by afferents stimulated by the satiety hormone (11). Cannabinoids were shown to regulate appetite in the guinea pig in part through both presynaptic and postsynaptic actions on anorexigenic POMC neurons (12).

Several neurotransmitter substances and neurochemicals like glutamate (Glu), catecholamines, 5-HT, acetylcholine; numerous neuropeptides such as substance P (SP), neurokinin A, vasoactive intestinal peptide, calcitonin gene-related peptide (CGRP), somatostatin (SS), and CCK and other neuroactive molecules (e.g. nitric oxide), have been identified in the neurons of the nodose ganglia (13). In the current study the interaction between cannabinoids and CCK was investigated by double labeling the neurons for both c-Fos and TH positive immunoreactivity.

c-Fos functions by rapidly altering gene transcription in response to cell surface signals, either positively or negatively (14). Most neurons express little or no c-Fos under baseline conditions (15). TH is involved in the conversion of phenylalanine to dopamine. As the rate-limiting enzyme in the synthesis of catecholamines, tyrosine hydroxylase has a key role in the physiology of adrenergic neurons. TH is regularly used as a marker for catecholaminergic neurons.

The aims of the present study were to demonstrate that the injection of rats with CCK intraperitoneally (IP) influences neuronal activity in NTS as assessed by induction of c-Fos expression and that ICV administration of cannabinoid agonist CP55,940 inhibits CCK effect, and secondly that this suppression of CCK-induced c-Fos protein expression in NTS by CP55,940 will be decreased by pretreatment of the rats with cannabinoid antagonist SR141716. Lastly the NTS neurons involved in the interaction of cannabinoid agonist CP55,940 administered ICV and CCK injected (IP) will be identified using fluorescence and light microscopy. The difference in the distribution of catecholaminergic neuronal subpopulations in NTS that are positive for both c-Fos and TH immunoreactivity will be determined.

Materials and Methods

Chemicals and Reagents

CCK8 peptide was purchased from New England Nuclear, Boston, MA and cannabinoid agonist CP55,940 was from Tocris Cookson, Ellisville, MO. Cannabinoid antagonist SR141716 was obtained from National Institute on Drug Abuse, Rockville, Maryland. Primary antibodies for c-Fos and TH were from Oncogene Sciences Inc, Uniondale, NY and Affinity Bioreagents, Golden, CO respectively. Cy3 antibodies were purchased from Molecular Probes, Eugene, OR and diaminobenzidine (DAB) kit was from Vector labs, Burlingame, CA. Standard laboratory rat chow pellets were obtained from Purina 5012, Purina Mills, St. Louis, MO and water were available ad libitum. All protocols were approved by the University of Georgia Institutional Animal Care and Use Committee.

Experimental setup

The tissues used for this study were obtained from the same animals that were used for the earlier study (Chapter 2).

Immunohistochemistry

The tissues were double labeled for c-Fos and TH immunohistochemistry. The tissues were first stained for c-Fos immunoreactivity as described in Chapter 2. After staining for c-Fos immunoreactivity the tissues were thoroughly washed once every 20 minutes for 3-4 times. The tissues were then processed for TH immunoreactivity. Briefly, the sections were pre-incubated in 0.3 % triton-X 100 (PBS-TX) with 4% normal donkey serum (NDS) for 2 h at 4°C. The sections were then incubated in monoclonal antibodies against TH (1:3000 rabbit anti-TH in PBS-TX with 1 % NDS) for 48 - 72 h, followed by incubation with the, Cy3 conjugated rabbit anti-donkey secondary antibody (1:3000) for 1 h. Sections were thoroughly washed three times with PBS-TX and rinsed with distilled water. Sections were then mounted on gelatin coated slides and air dried. Once dry the sections were washed in a series of ethanol solutions for dehydration and xylene for delipidation. The sections were then cover slipped using Depex histological mounting medium (Fisher Scientific, Fair Lawn, NJ). Sections were viewed using a Nikon fluorescence microscope and the images were taken with a Ziess Axioplan 2 imaging microscope using SPOT Basic software and quantified using Image-Pro Plus software (Media Cybernetics, Inc, Bethesda, MD) A stereotaxic rat brain atlas was used to determine the location of the NTS in each section of tissue (16). A region of interest was drawn around the NTS, and all activated neurons in the NTS region of interest were counted. Neurons were considered immunopositive by the intensity of staining and size. Representative sections were chosen, corresponding to different regions of the NTS. The numbers of labeled neurons per

section were summed for each region for each rat; this value was used in subsequent statistical analyses.

Statistical analysis

Statistical analysis was performed using a one-way ANOVA by counting the number of cells in the NTS showing both c-Fos and TH immunoreactivity. A minimum of ten sections were counted per each animal. The counts used in statistical analysis represent the average number of NTS neurons showing positive immunoreactivity for c-Fos and TH. A value of $P < .05$ was used to establish statistically significant differences.

Results:

The data in Fig.1 show that animals treated by ICV infusion of CP55,940, and IP injection of CCK did not show any statistically significant difference in the tyrosine hydroxylase positive cells showing c-Fos immunoreactivity between the two treatment groups. The number of TH positive neurons showing c-Fos immunoreactivity in the NTS of CP/CCK treated rats was 39 ± 6 ; mean \pm SEM, while the counts in the saline/CCK treated rats was NTS 42 ± 9 ; mean \pm SEM ($P < 0.05$). The counts of TH positive neurons showing c-Fos immunoreactivity in the NTS of the animals that were pretreated with cannabinoid antagonist SR141716 followed by CP55,940 (ICV) and CCK (IP) were 13 ± 1 . There was no significant difference between this treatment group and the rats treated with saline/CP/CCK (7 ± 1), SR/saline/CCK (13 ± 1), saline/saline CCK (15 ± 4) (Figure 2). The representative photomicrographs in Figure 3 shows the double labeling immunofluorescence for TH and c-Fos in the NTS of rats treated with SR141716, CP55,940 and CCK in the respective treatment groups.

Discussion:

The aim of this study was to identify the specific subpopulations of CCK-activated neurons in NTS that facilitate CCK-cannabinoid interaction. Intraperitoneal administration of CCK octapeptide (CCK-8S) has been shown to induce changes in neuronal activity in the NTS and AP, sensory parts of the dorsal vagal complex (DVC), and in the paraventricular nucleus of the hypothalamus (PVN), as determined by activation of c-Fos expression (17). In a different study, c-Fos positive neurons induced by CCK were positive for corticotropin-releasing factor immunoreactivity, which were mainly localized in the ventral part of the dorsomedial hypothalamic nucleus (DMH), and encircled in a network of tyrosine-hydroxylase-immunoreactive positive fibers. Thus in addition to the PVN, peripheral CCK increases neuronal activity in the DMH suggesting a possible role in this hypothalamic nucleus in the satiating effect of the peptide (18). CCK was shown to abolish ghrelin-induced food intake through dampening increased ARC neuronal activity (19). Our results showing c-Fos and TH positive immunoreactivity induced by the peripheral administration of CCK in NTS and AP of rats is in agreement with the above mentioned studies.

The behavioral effects of cannabinoids are mediated by CB1, the cannabinoid receptors, (20). While CCK is considered to be a satiety factor (21), CB1 was proposed to induce feeding behavior (22). Thus, the two systems act in an antagonistic fashion. High degree of coexpression of CB1 with CCK suggested a possible functional cross-talk between the two systems. On the other hand, the interaction of CB1 and CCK was also thought to regulate CCK synthesis by cannabinoids (23). In this study CB1 receptor agonist CP55,940 inhibited CCK-stimulated c-Fos activation in NTS while CB1 antagonist SR141716 reversed this effect, confirming the possible cross-talk between cannabinoid and CCK systems.

The NTS receives dense terminations from cranial and visceral afferents, but little is known about which NTS neurons are involved or what mechanisms are responsible for the controlled regulation of both short-term and long-term feeding behavior, body weight and energy homeostasis. Glutamate, which is an excitatory neurotransmitter is released by the primary afferent fibers, and activates non-N-methyl-D-aspartate (non-NMDA) receptors on second-order neurons in the NTS. Both NMDA and non-NMDA receptors coexist on the same second-order NTS neurons and mediate primary visceral afferent transmission in the NTS (24). Rinaman et al., (10) showed that in the dorsomedial medulla, 51% of catecholaminergic neurons and 39% of neurons positive for neuropeptide Y were activated by peripheral administration of CCK (10). CB1 is expressed in various neocortical neuronal populations, including glutamatergic neurons and widely mediates endocannabinoid effects on glutamatergic and GABAergic transmission to modulate cortical networks (3).

Results from our immunohistochemical studies have shown c-Fos immunoreactivity in TH-positive neurons in NTS. So these findings are in agreement with earlier results showing CCK activation of catecholaminergic neurons in NTS. However, in the current study we did not see any statistically significant difference in the catecholaminergic neurons activated by CCK between the treatment groups. The plausible explanation is that the interaction between CCK and cannabinoids might be facilitated by the other neuronal subpopulations activated by CCK. Rinaman et al., (1993) showed that the catecholaminergic neurons constitute of only 51% of the neurons activated by CCK. Therefore the other neuronal subpopulations like NPY neurons, GABAergic neurons or glutamate neurons might be facilitating this interaction. Further immunohistochemical studies are needed to identify the specific neurons in NTS.

Figures

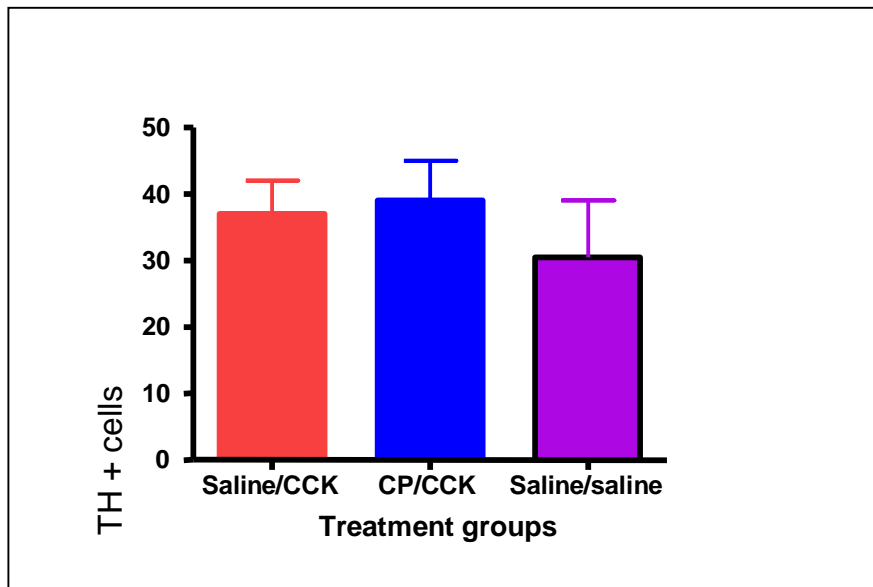


Figure 1. Number of tyrosine hydroxylase and c-Fos immunoreactive neurons (mean±SE) in NTS of rats administered with saline (ICV) and CCK (IP); CCK (IP) and CP 55,940 (ICV); saline (IP)/saline (ICV).

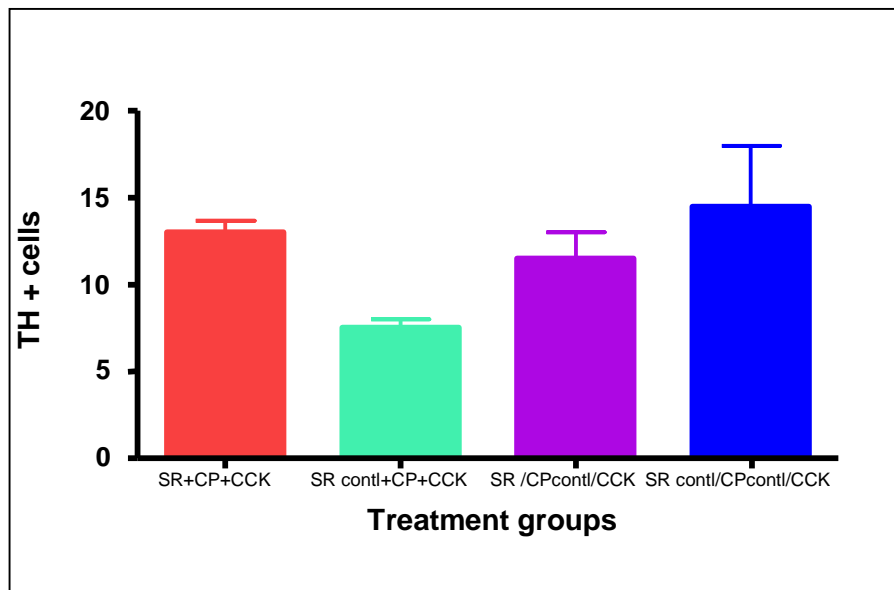
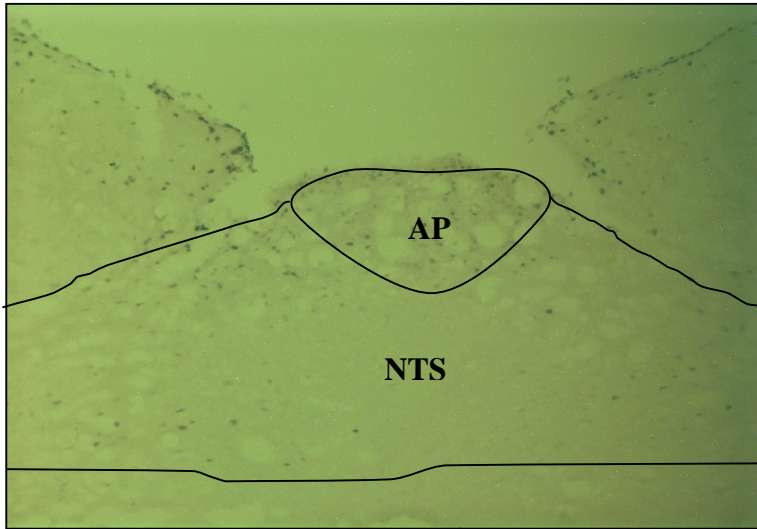


Figure 2. Number of tyrosine hydroxylase and c-Fos immunoreactive neurons (mean±SE) in NTS of rats administered with different treatment groups comprising of CB1 receptor agonist CP55,940, CB1 antagonist SR141716, CCK and saline.

A.



B.

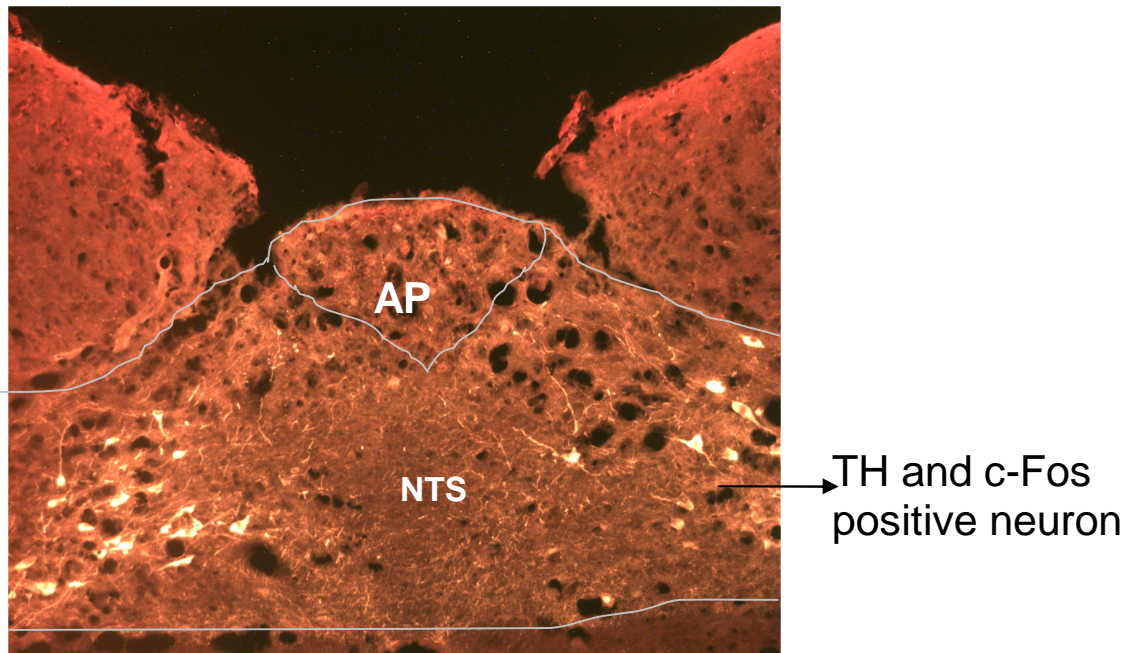


Figure 3. Photographs depicting c-Fos and tyrosine hydroxylase immunoreactive positive neurons in the NTS of rats detected and counted using immunohistochemistry and image analysis. Sections of the dorsomedial region of hindbrain illustrating the areas of NTS where c-Fos positive neurons were counted. A. Section of hind brain showing the NTS and AP area with c-Fos staining. B. Section of hindbrain showing the NTS and AP area with TH immunoreactivity.

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Inhibition of cholecystokinin induced satiety by cannabinoid agonist CP 55,940

(Behavioral study)⁵

⁵ Gaddam DR, Freeman KA, Edwards GL

Abstract

Cannabinoid agonist CP55,940 when injected into the 4th ventricle has been shown to increase food intake, especially, the consumption of highly palatable foods. It has been previously shown from our lab that the intracerebroventricular injection of cannabinoid agonist CP 55,940 increased the consumption of sweetened condensed milk. CP 55,940 increased milk intake at doses in the microgram range when injected in the lateral ventricle, but only required nanogram doses when injected into the fourth ventricle to attain the same effect. Cholecystokinin (CCK) is a well know satiety signal from the gut that is transmitted by vagal afferents that activate the neurons in the nucleus of solitary tract (NTS). Intraperitoneal administration of cholecystokinin was found to increase c-Fos immunoreactivity in the neurons of NTS and area postrema (AP). So we postulated that the effect of IP injection of CCK can be attenuated by the ICV administration of CP 55,940 into the 4th ventricle and thus, increase the consumption of palatable food. Cannulated rats were trained to consume vanilla wafer cookies. Cookie intake was measured after ICV injection of CP 55,940 and/or IP injection of CCK. Cookie intake was recorded for a total of 3 hours. There appears to be an increase in consumption of the cookies in the first 30 minutes after CP 55,940/CCK when compared to saline/CCK group, however; this increase was not statistically significant. Some of the reasons believed to be responsible for these results are, the high dose of CP55,940 might have caused some motor effects affecting the mobility and balance of the rats. Other possible reasons might be the smaller sample size per each treatment group, high dose of CCK and technical problems leading to improper administration of the test drugs.

Key words: CP 55,940, CCK, NTS

Introduction

Appetite is regulated by a complex system of central and peripheral signals that interact with one another to modulate the individual response to food consumption. Peripheral regulation includes satiety signals from the gut and the hormonal signals from adipose tissue. The central control is accomplished by several effectors, including the neuropeptidergic, monoaminergic and endocannabinoid systems [1]. Satiety signals, including cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1) and peptide YY (PYY), originate from the gastrointestinal (GI) tract during a meal and, reach the nucleus of solitary tract (NTS) in the caudal brainstem through the vagus nerve. From NTS afferent fibers project to the arcuate nucleus (ARC), where satiety signals are integrated with adiposity signals, like leptin and insulin, and also with several hypothalamic and supra-hypothalamic inputs, creating a complex neuronal network which finally results in the individual response to a meal [1]. The brain-gut peptide cholecystokinin (CCK) inhibits food intake following peripheral or site directed central administration. Peripheral exogenous CCK inhibits food intake by reducing the size and duration of a meal. Antagonist studies have demonstrated that the actions of the exogenous peptide mimic those of endogenous CCK. Antagonist administration results in increased meal size and meal duration. The feeding inhibitory actions of CCK are mediated through interactions with CCK-1 receptors [2]. The ability of exogenously administered cholecystokinin (CCK) to inhibit food intake in rats was originally demonstrated by Gibbs et al [3]. CCK reduced meal size and meal duration and resulted in an earlier appearance of a behavioral sequence of satiety similar to that seen following ingestion of a normal size meal [4]. Work in multiple laboratories on the satiating effect of CCK-8 in adult rats [5], has now demonstrated that exogenous peripheral administration of CCK results in dose-related suppression of short-term food intake in a variety of species and

in a variety of experimental situations [6, 7]. The availability of CCK receptor antagonists provided the ability to critically assess the physiological significance of endogenous CCK in the controls of meal size. In a variety of testing paradigms across multiple species, CCK antagonists with specificity to the CCK-1 receptor subtype have been shown to increase food intake [6, 8, 9]. Results with CCK antagonists in human subjects have been mixed. Studies have demonstrated small but significant increases in caloric intake in response to the CCK-1 antagonist loxiglumide [10]. These increases corresponded to decreased reports of fullness and increased reports of hunger [10]. The c-Fos immunohistochemical technique [11] has been used to identify sites in the central nervous system of adult rats that are stimulated by the peripheral injection of CCK-8. Intraperitoneal administration of CCK-8 increased the number of c-Fos- like immunoreactive cells in several portions of the NTS and in forebrain nuclei including the supraoptic (SON), paraventricular (PVN) nuclei, and the central nucleus of the amygdala (CeA) [12-14]. CCK-elicited vagal activation is processed at the level of the caudal brainstem. Integration at this site is sufficient for limiting meal size as demonstrated by the ability of CCK-8 to inhibit food intake in adult decerebrate rats [15].

Recent studies suggest that the endocannabinoid system modulates feeding. Despite the existence of central mechanisms for the regulation of food intake by endocannabinoids, evidence indicates that peripheral mechanisms may also exist. It is generally thought that the hyperphagic actions of cannabinoids are mediated by CB1 receptors located in brain circuits involved in the regulation of motivated behaviors [16]. Studies have shown that CB1 receptor widely mediates endocannabinoid effects on glutamatergic and GABAergic transmission to modulate cortical networks [17]. CB1 activation depressed GABAergic IPSCs elicited by cholinergic agonists in pyramidal neurons. This effect was strongly reduced by the CB1 antagonist AM 251. So it is

believed that CB1 mediates cannabinoid effects on other cell types, including pyramidal glutamatergic neurons. CB1 appears to play a major role to modulate cortical activity by acting on both glutamatergic and GABAergic neurons [17]. The presynaptic activation of CB1 receptors is linked to inhibition of N- or P/Q-type voltage-gated calcium channels involved in vesicular release [18]. CB1 receptors are found on nerve terminals innervating the gastrointestinal tract [19, 20], which are suggested to be involved in mediating satiety signals that originate in the gut [21]. It was shown that systemically administered cannabinoid agents (both agonists and antagonists) affect food intake predominantly by engaging peripheral CB1 receptors localized to capsaicin-sensitive sensory terminals [22]. The expression of the early gene c-Fos on hypothalamic and brainstem areas regulating food intake after both the peripheral administration of either CB1 agonists and antagonists [23] and the acute administration of peripherally acting satiety modulators such as gastrointestinal hormones [23] or feeding inhibitors such as OEA [23] support the peripheral actions of cannabinoids on food intake. In addition to the immunohistochemical studies mentioned in the earlier chapters, the effect of administration of cannabinoid agonist CP55,940 (ICV) and CCK (IP) on palatable food intake in rats was also investigated. We hypothesized that the ICV administration of CP 55,940 inhibits the satiety effect of CCK and increases the consumption of palatable food.

Materials and methods:

Animals

The animals used for this study are the same animals used for immunohistochemistry in Chapter 1. Rats weighing 200–250g at the start of each experiment were housed in shoe box cages and were maintained on a 12:12-h light–dark cycle in a temperature-controlled

environment (temperature 22 ± 2 °C; humidity 50–60%). Food and water were provided ad libitum.

Chemicals and Reagents

CCK8 peptide was purchased from New England Nuclear, Boston, MA and cannabinoid agonist CP55,940 was from Tocris Cookson, Ellisville, MO. Kroger brand vanilla wafer cookies were used as the palatable food. Standard laboratory rat chow pellets (Purina 5012, Purina Mills, St. Louis, MO) and water were available ad libitum. All protocols were approved by the University of Georgia Institutional Animal Care and Use Committee.

Experimental Procedures

Surgery and cannula placement

A 22-gauge stainless steel cannula (Plastics One, Roanoke, VA) was implanted in the fourth cerebroventricle. Each rat was anesthetized with a cocktail of ketamine–acepromazine–xylazine (50:3.3:3.3 mg/kg im). The dorsum of the head and neck was clipped and vacuumed to remove loose hair. The anesthetized rat was placed in a stereotaxic apparatus with the neck flexed. The surgical area was cleaned with chlorhexidine to disinfect the skin. An incision was made in the skin over the skull, and the periosteum was removed from the calvarium. Burr holes were drilled for the placement of two anchor screws and the cannula. The coordinates used for placement of cannula into the fourth ventricle were 2.0 mm rostral to the parietal–occipital suture on the midline and 7.5 mm ventral from dura, with the skull level between lambda and bregma [24]. Dental acrylic was used to embed the screw heads and cannula, thereby fixing the cannula in place. When the acrylic was dry, a 30-gauge obturator was placed in the cannula. A 7- to 10-day postsurgical recovery period was allowed for body weights to stabilize prior to training the rats for intracerebroventricular injections.

Histological verification of cannula placement was performed in all rats by injecting methylene blue dye after euthanizing the animals, and any rat with improperly placed cannula was omitted from data analysis.

All drug trials were set up using a random design with CP55,940 and CCK depending on the experiment. Rats were intracerebroventricularly injected with CP55,940 or vehicle using a Gilmont micrometer syringe fitted to an injector that extended 1 mm beyond the cannula tip. 50% DMSO and 50% saline was used as a diluent for CP55,940. Fifteen minutes after the first injection the rats were injected with CCK or saline IP. It was previously reported from our lab that rats intracerebroventricularly (ICV) injected with a solution containing 50% DMSO and 50% saline did not significantly alter the intake of sweetened condensed milk compared to rats injected with saline alone (data not shown). For this reason, control animals in these studies received saline alone.

Feeding trials

For the feeding trials, the animals were transferred from plexiglass cages to hanging steel cages. One rat was placed in each cage. Each rat was offered three cookies at a time. The weight of three cookies was recorded at the beginning of the trial. Thin card board sheets were placed beneath the hanging cages to collect the crumbs. The amount of cookie consumed was measured every half an hour by taking out the cookies remaining in the cage and also the crumbs from the card board sheet and subtracting their total weight from the initial weight. At least one new cookie was offered after every weight recording. Cookie intake was recorded 4 times during the two hour period. After the completion of the feeding trial rats were transferred back to the plexiglass cages. These were done on alternate days.

Results:

The results indicated that animals injected with saline (ICV and IP) consumed 4.4 ± 0.5 , 2.4 ± 1.1 and 1.7 ± 0.3 grams at 30, 60 and 90 minutes respectively. Injection of CCK (IP) and saline (ICV) decreased cookie intake to 3.45 ± 0.6 grams after 30 min but was not statistically significant. At the same time this effect of CCK (IP) appeared to be antagonised by CP (ICV), where rats consumed significantly higher levels of cookies 5.0 ± 0.3 grams. Sixty minutes after injection there was no difference between the three treatment groups. At 90 minutes the rats that received saline alone consumed 1.7 ± 0.3 gm, CCK (IP) and CP (ICV) consumed 4.2 ± 0.7 grams and CCK (IP) and saline (ICV) consumed 3.6 ± 0.05 grams which were not statistically significant (Figure 1).

Discussion:

It is well accepted that the brain plays a major role in the modulation of appetitive behavior. Feeding studies investigating the role of cannabinoids in appetitive responses have employed various methods of peripheral administration of the cannabinoid or cannabinoid antagonist. But, direct injection of cannabinoids into the brain is an appropriate method to study the effect of cannabinoids on feeding systems. Numerous studies suggest that the hindbrain plays an important role in the control of ingestive behavior (1).

In the current study, it appears that CP55,940 suppressed the satiating effect of CCK and increased the consumption of cookies during the 30 minutes after injections. Although the same trend was not noticed at later time points, the overall amount of cookies consumed decreased. But there was no statistically significant decrease in cookie consumption noticed between the different treatment groups. The reasons might be the dose of CP55,940 injected into fourth ventricle. Earlier reports showed that rats injected with the 1 ng dose of CP55,940 drank

significantly more sweetened condensed milk at 1, 2, and 3 h (2). CP55,940 is injected at 0.5 μ g dose per animal in this study. Significant motor effects were noticed after 30 min this might have compromised the ability of rats to hold the cookies and chew on them. Also the results show that there was no visible satiety effect of CCK. This might be due to the dose of CCK injected and the number of the rats. The experiment should be repeated with different doses of CP55,940 and CCK to demonstrate the suppression of CCK's satiety effect on feeding behavior of rats.

Figures:

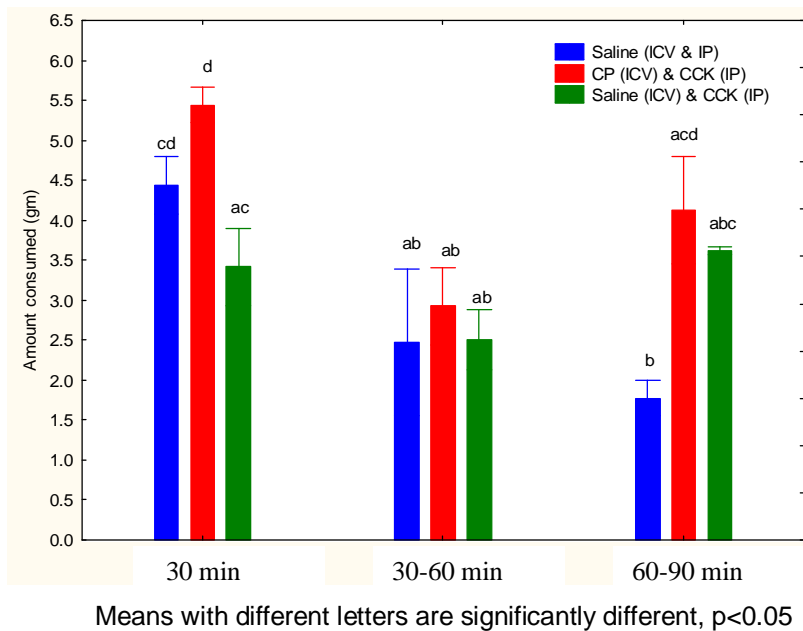


Figure 1: Intake of cookies (gm, mean \pm S.E.M.) in rats ($n=12$) injected with CP 55,940 (ICV) and CCK (IP) or saline into the fourth ventricle and at time 0 and presented with vanilla wafer cookies 15 min after injection. CP 55,940-injected rats ate significantly more cookies than the rats injected with CP and CCK in the first 30, and there was no significant difference in the cookie consumption at 60, and 90min between the different treatment groups after presenting cookies. ($P < .05$).

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Summary and Conclusions

Several neurotransmitters and neuromodulators act in coordination at different regions of brain like forebrain, arcuate nucleus of the hypothalamus, hindbrain nuclei and peripheral ganglia like the nodose ganglion, to control feeding behavior and energy expenditure. The dorsomedial medulla is an important center which acts as the convergence point for the integration and bidirectional flow of information. Sensory afferent information flows towards the higher regions of the brain and the efferent motor information towards the periphery (1). Studies with decerebrate rats demonstrated that, in the absence of connections between the forebrain and caudal brainstem, the isolated caudal brainstem is sufficient to mediate many aspects of the short term feeding and energetic response to starvation that are attributed to hypothalamic signal processing (2). Anatomical, physiological, and behavioral data suggest that the ingestive behavioral outputs are modulated by neural signaling in the dorsal vagal complex (DVC), comprising the nucleus of the solitary tract (NTS), area postrema (AP), and dorsal motor nucleus of the vagus (3). Research on the control of food intake suggests that it is a complex process of mechanisms involving several neuroactive substances through which food intake is regulated. Cholecystokinin (CCK) and endocannabinoids are two of the neuroactive substances that are well established to play an important role in the regulation of food intake. Cannabinoids have been shown to influence food intake, and until recently, the neural pathways mediating these effects have not been clearly understood. Cholecystokinin (CCK) is a well know satiety signal from the gut that is transmitted by the vagal afferents and has been shown to activate the neurons in the nucleus of solitary tract (NTS) and area postrema (AP) (4). CCK reduces food intake by

acting at CCK-1 receptors on vagal afferent neurons. Gastric and intestinal vagal afferents that express receptors for cholecystokinin (CCK) also express CB1 receptors (5). Effect of endogenous cannabinoids on appetite may be partially mediated by vagal afferent neurons. CCK, which is released from the gut by food, is shown to decrease CB1 receptor expression in vagal afferent neurons (5). It has been established that endogenous CCK participates in the short-term regulation of food intake thus; it is possible that the inhibition of this satiety signal would contribute to an increase intake of palatable, high caloric food. It has also been shown that the administration of exogenous cannabinoids increases intake of highly palatable food (6). CB1r agonist CP55,940 decreased or inhibited the entry of extracellular calcium into isolated vagal afferent neurons collected from nodose ganglion (unpublished data). These studies suggest that cannabinoids suppress CCK's actions in the brain. Earlier studies from our lab have also shown that intracerebroventricular (ICV) administration of cannabinoid agonist, CP55,940 into the 4th ventricle increased the consumption of sweetened condensed milk. Neurons in the NTS and AP are shown to express c-Fos protein following intraperitoneal (IP) injection of CCK (7). Our first hypothesis is that cannabinoids decrease CCK activation of c-Fos in NTS and AP neurons. To test this hypothesis cannulated rats were given CP55940 (0.5µg/5µl/animal) via the 4th ventricle and were administered CCK (5µg/kgB.Wt, IP), 15 minutes after CP55940 injection. Our results showed that CP55940 suppresses the c-Fos expression activated by CCK. The c-Fos counts in the CP/CCK treated rats, (NTS 69 ± 19 , AP 30 ± 6 ; mean±SEM) were significantly lower than the counts in the saline/CCK treated group (NTS 371 ± 94 ; AP 150 ± 47 ; mean±SEM) ($P < 0.05$). Results using the cannabinoid 1 receptor (CB1r) antagonist SR 141716, CP55,940 and CCK showed that the inhibitory effect of CP55,940 on c-Fos activation of CCK was effectively blocked using the SR141716. The c-Fos counts in the SR/CP/CCK treated rats were

significantly higher than those from rats treated with saline/CP/CCK (104.3 ± 40.12 vs 24.29 ± 6.08). From the results of our study we were also able to confirm that CCK induced c-Fos like immunoreactivity in the NTS and AP neurons. Our results also showed that cannabinoid agonist CP 55,940 suppresses the CCK induced c-Fos immunoreactivity in the NTS and AP neurons. Our study with the cannabinoid antagonist SR141716 decreased the inhibitory effect of CP55,940 on neuronal activation of CCK suggesting that this effect is mediated through the CB1r. We also did a behavioral study to show the suppression of CCK activity of CP55, 940 on cookie consumption. We postulated that the effect of IP injection of CCK can be blocked and reversed by the ICV administration of CP55,940 into the 4th ventricle and thus increase the consumption of palatable food (vanilla wafer cookies in our case). Cannulated rats were trained to consume vanilla wafer cookies after ICV injection of CP 55,940 and IP injection of CCK. Cookie intake was recorded for a total of 3 h. We did not see any statistically significant increase in the consumption of cookies after the ICV injection of CP 55,940. There was an increase in consumption of the cookies in the first 30 minutes which was not statistically significant. Not much change in the cookie consumption was noticed latter. We believe that the high dose of CP55,940 might have caused some motor effects affecting the mobility and balance of the rats. The motor effects of CP55,940 were clearly visible in some of the rats. The results from our behavioral study did not show any statistically significant differences in the cookie consumption of the different treatment groups. It has been demonstrated that only certain neuronal subpopulations like catecholaminergic neurons, NPY neurons, Glutamate neurons and GABAergic neurons are activated by peripheral administration of CCK (8). Having proved that CP55,940 significantly suppresses the CCK activity in the NTS and AP neurons, our next objective was to identify the sub population of the NTS neurons that are involved in this

mechanism. We started our study to see if these neurons are catecholaminergic neurons. Our hypothesis for this study is that the affect of cannabinoids on CCK activation of c-Fos in NTS and AP neurons involves catecholaminergic neurons. The hindbrain tissues used in the first study (Chapter1) were also used for the double labeling using c-Fos and TH antibodies. Our results from this study also confirmed that CP55,940 suppresses the c-Fos expression activated by CCK. The c-Fos activity was found in TH positive cells but there was no statistically significant difference in the number of TH positive neurons showing the c-Fos immunoreactivity between the two treatment groups. The number of TH positive neurons showing c-Fos immunoreactivity in CP/CCK treated rats, (NTS 39 ± 6 ; mean \pm SEM) were not significantly different than the counts in the saline/CCK treated rats (NTS 42 ± 9 ; mean \pm SEM) ($P < 0.05$). Similarly the counts of TH positive neurons showing c-Fos immunoreactivity in the NTS of SR/CP/CCK treated rats (13 ± 1) were not significantly different from the rats treated with saline/CP/CCK (7 ± 1), SR/saline/CCK (13 ± 1), saline/saline CCK (15 ± 4).

Conclusion and future studies

We have confirmed that IP administration of CCK activates neurons in NTS and AP. We have shown for the first time with immunohistochemical studies that cannabinoid agonist CP 55,940 suppresses the neuronal activation of CCK. Further studies using a lower dosage of CP 55,940 are necessary to reproduce our immunohistochemical results in behavioral studies to actually see how CP 55,940 inhibits or reverses the satiation effect of CCK and increases food consumption. We have shown with our double labeled immunohistochemistry studies that the neurons involved in this cannabinoid-CCK interaction are not catecholaminergic neurons. Further doublelabeling immunohistochemistry studies are necessary to see if these neurons belong to other neuronal subpopulations like glutamate, NPY or GABAergic neurons.

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