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Role of Sensory Circumventricular Organs in the Function of Hormones Affecting Feeding and Energy Balance

by

Al-Shaimaa Faissal Fadel Ahmed

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Abstract

The subfornical organ (SFO) is a sensory circumventricular organ implicated in the regulation of fluid homeostasis and cardiovascular functions and characterized by the presence of receptors for a number of peripheral signals affecting food intake and energy balance such as leptin and cholecystokinin (CCK). CCK is an intestinal satiety hormone affecting short and long term energy balance. Given the importance of CCK in energy homeostasis and the presence of its receptors in the SFO, the objective of this thesis was to study the effect of CCK on the activity of the SFO using the area postrema (AP) and the nucleus of the solitary tract (NTS) as positive control regions. The effect of exogenous CCK-8 on the activity of the SFO neurons was examined using the functional activation markers c-Fos and p-ERK. CCK increased c-Fos immunoreactivity in the NTS and the AP as well as in the SFO compared to controls. Blockade of $CCKR_1$ reduced the effects of CCK in the AP and the NTS, but not in the SFO, while antagonism of $CCKR_2$ blocked CCK-induced SFO activation. Rats subjected to subdiaphragmatic vagotomy were used to study the contribution of the vagus nerve to CCK-induced effect on the SFO. It was found that CCK-induced activation of SFO was not dependent on an intact vagus, contrary to CCK-induced increase in c-Fos in the AP and the NTS which was abolished by vagotomy. Then, the effect of endogenous CCK was studied using meal-induced release of CCK which, similar to the effect of exogenous CCK, resulted in increased c-Fos activation. In a model of high fat diet (HFD) induced obesity, the effect of CCK on c-Fos expression in the AP and NTS was abolished. Surprisingly, HFD-fed rats experienced high basal c-Fos levels which made it impossible to investigate the effect of

CCK in the SFO. In conclusion, these results show that SFO is a potential target for the actions of exogenous and endogenous CCK. These actions are not dependent on the vagus nerve. HFD induces changes in SFO activity evident by increased basal c-Fos immunoreactivity within the SFO of HFD-fed rats.

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Dedication

To my Mom and my Dad

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List of Abbreviations

α-MSH	alpha-melanocyte-stimulating hormone
ACE	Angiotensin converting enzyme
AGRP	agouti-gene related protein
AngI	Angiotensin I
AngII	Angiotensin II
ANOVA	Analysis of Variance
AP	Area postrema
ARC	Arcuate nucleus
AT1R	Angiotensin receptor subtype 1
AT2R	Angiotensin receptor subtype 2
BBB	blood brain barrier
CART	cocaine- and amphetamine-regulated transcript
CaSR	Ca^{++} sensitive receptor
CB_1	endocannabinoid receptor
CCK	Cholecystokinin
CCK-8S	sulphated form of CCK-8
$CCKR_1$	Cholecystokinin receptor subtype 1
$CCKR_2$	Cholecystokinin receptor subtype 2
CNS	central nervous system
CVOs	circumventricular organs
db/db mice	leptin receptor mutant mice
DBI	diazepam-binding inhibitor
DIO	diet-induced obese

DMH	dorsomedial hypothalamic nucleus
DMN	dorsal motor nucleus of the vagal nerve
DMV	dorsal motor vagus
DR	diet resistant
ENS	enteric nervous system
ERK	Extracellular signal -regulated kinase
GABA	γ-aminobutyric acid
GHSR	Growth-hormone secretagogue receptor
GI	gastrointestinal
GIP	gastric-inhibitory peptide
GLP	Glucagon like peptide
HFD	High fat diet
I cells	intestinal cells
i.p.	intraperitoneal
icv	Intracerebroventricular
IL-1β	interleukin-1β
IP3	inositol triphosphate
JAK/STAT	Janus Kinase/ Signal Transducers and Activators of Transcription
LCRF	luminal CCK-releasing factor
LH	Lateral hypothalamus
LPS	lipopolysaccaride
M1	muscarinic cholinergic receptor
MAPK	mitogen activated protein kinase
MC4	melanocortin receptor 4

NPY	Neuropeptide Y
NTS	Nucleus of the solitary tract
ob/ob mice	leptin protein mutant mice
ObR	leptin receptor
OLETF	Otsuka Long Evans Tokushima Fatty rats
OVLT	Organum vasculosum of the lamina terminalis
OXM	Oxyntomodulin
PBS	Phosphate buffered saline
pERK1/2	phosphorylated extracellular signal regulated kinase 1 and 2
PFA	Paraformaldehyde
PKC	protein kinase C
PLC	phopolipase C
PLD	phospholipase D
POMC	Pro-opiomelanocortin
p-STAT	phosphorylated-signal transducer and activator of transcription 3
PVN	Paraventricular nucleus
PYY	Peptide tyrosine tyrosine/peptide yy
RAS	Rennin angiotensin system
SBTI	Soybean trypsin inhibitor
SFO	Subfornical organ
TNF-α	tumor necrosis factor-α
Vgx	Vagotomized/ subdiaphragmatic vagotomy
VMH	ventromedial hypothalamic nucleus

Chapter 1 General Introduction

The purpose of this chapter is to give a background introduction on the gut-brain axis and its components as well as its role in feeding and energy balance. This will be followed by a description of the different pathways through which the gut interacts with the brain with special emphasis on sensory circumventricular organs; characterized by a lack of blood brain barrier, a feature that permits these structures to be in direct contact with circulating factors. The role of the sensory circumventricular organs in the control homeostatic functions will be briefly discussed followed by a more detailed review of the current knowledge about their role in metabolic functions and energy homeostasis.

1.1 Gut-brain axis.

The gut brain axis represents the bidirectional signaling between the gastrointestinal (GI) tract and the brain. It is important for maintaining homeostasis and involves nervous and humoral signals arising from the gut and acting directly or indirectly on the brain [1]. The central autonomic pathways of the brain integrate this information and appropriate outputs are generated. These can be behavioral, psychological, humoral or neural reflex outputs [2]. The brain gut axis is involved in the control of digestion, host defense and many homeostatic regulatory pathways including those that control inflammation (e.g. vagal anti-inflammatory pathway[3]) and energy balance [1]. In this thesis, I will focus on one aspect of the brain gut axis used in the control of energy balance.

The GI tract receives innervation from the parasympathetic (vagal and pelvic nerves) and the sympathetic (splanchnic nerve) divisions of the autonomic nervous

system. Vagal motor neurons provide input to the stomach, small intestine and proximal colon. Excitatory vagal input mediate vago–vagal motor reflexes and secretion of gastrin as well as the release of histamine and serotonin from enterochromaffin cells [1]. The sympathetic innervation of the GI tract is divided into postganglionic vasoconstrictor neurons, secretion inhibiting neurons and motility inhibiting neurons. The overall effect of adrenergic outflow to the gut is inhibitory, slowing GI transit and inhibiting secretions. This inhibitory effect is largely accomplished by inhibitory modulation of cholinergic transmission and by a stimulatory effect on smooth muscle of the sphincters [4]. In addition to an extrinsic innervation of the gut, the enteric nervous system (ENS), originally described by Langley [5] as the third division of the autonomic nervous system, provides an intrinsic neural control of the GI tract. The ENS, often described as "the second brain" [6], is characterized by plasticity and autonomy with respect to the central nervous system (CNS) .

Afferent fibers of the gut brain axis transmit signals to the CNS from different sensory endings located in the GI tract. These sensors detect different types of stimuli such as mechanical (e.g. gastric distension), chemical (e.g. nutrients) and hormonal (gut hormones) as well as inflammatory mediators [7, 8]. Vagal afferents have their cell bodies in the nodose ganglia and terminate in the nucleus of the solitary tract (NTS). The NTS displays a viscerotopic organization for innervations originating from the esophagus and the stomach (rostral-lateral), stomach (caudal-medial) and from the intestine (central and rostral). Information reaching the NTS is then integrated and conveyed to the dorsal motor nucleus of the vagal nerve (DMN) located adjacent to the NTS.

Connections between the NTS and DMN complete the vago-vagal reflex pathways to control functions of the digestive system [9]. Some of the signals reaching the NTS are transmitted to higher brain centers such as hypothalamic nuclei (e.g. paraventricular nucleus; PVN and arcuate nucleus; ARC) as well as to the amygdala and the ventral hypothalamus [10].

In addition to the neural component of the gut brain axis, there are a number of signaling molecules released from the GI tract which play an important role in the gut brain axis by acting either to influence the activity of the vagal pathway or by acting as hormones released in the circulation to interact directly with the CNS [11]. Examples of these hormones are ghrelin, cholecystokinin (CCK), peptide YY (PYY), glucagon-like peptides (GLP), and oxyntomodulin (OXM) which will be discussed briefly in the following section. Figure 1.1 summarizes different pathways of gut-brain communications.

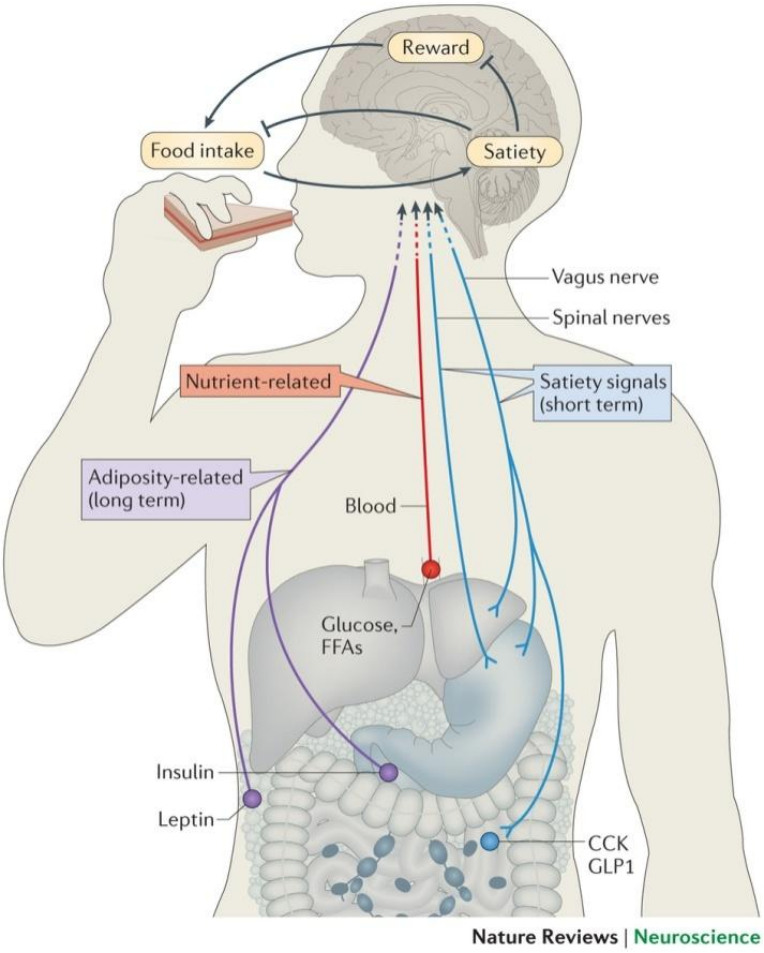

Figure 1.1: Gut-brain axis and control of food intake.

A diagram showing different pathways for the gut brain axis which includes the vagus nerve, gut related and nutrient related signals as well as signals originating from adipose tissue. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Neuroscience [12], Copyright (2014, appendix A).

1.2 Control of food intake.

Food intake is a complex behavior which involves integrative coordination between multimodal signals originating from the periphery and numerous regions within the CNS. The decision for initiation or cessation of feeding depends mainly on inputs from paracrine and endocrine systems which provide information about the energy status of the body. Failure to adjust the amount of food consumed leads to a status of either a positive or a negative energy balance. A positive energy balance leads to increased energy storage in the form of fat and the subsequent development of obesity. Obesity is considered a chronic disease due to its high risk of complications [13]. It leads to serious health problem with complications including diabetes [14, 15], cardiovascular diseases, stroke [16, 17] and cancer [18]. A recent study has shown that at least 18 comorbidities can be directly linked to obesity. In this study, the authors estimated the total direct costs of being overweight and obese to be $6.0 billion, accounting for 4.1% of the total health expenditures in Canada in 2006 [19].

Achieving a balance between energy input and output is difficult. However, the brain acts as a “homeostat” that senses, interprets and acts toward restoring optimum weight according to the body weight set point [20, 21]. In fact, there are different ways by which optimum weight is maintained. Body weight is maintained through the interplay between two central neural pathways: homeostatic and non-homeostatic. The homeostatic pathway deals with the internal environment and involves regulation of feeding and energy expenditure according to circulating nutrients and regulatory hormones. On the other hand, the non-homeostatic (hedonic) pathways deal with the

external environment and are affected by the availability and presentation of food and are mostly controlled by the brain reward centres (cortico-limbic system)[22]. Both homeostatic and non-homeostatic systems work as energy balance control systems that corrects for any “error” either in a positive or a negative manner, although the hedonic system has the ability to override the homeostatic pathways in controlling food intake. In other words, in a state of negative energy balance, these systems tend to enhance feeding and decrease energy expenditure. If a positive energy balance (i.e., more energy in than out) is maintained for an extended period of time, homeostasis will be disrupted and obesity will result in susceptible individuals [23].

Since the 1950s, lesion studies have shown the crucial role of the hypothalamus in the control of feeding and energy balance [24-27]. Within the hypothalamus, ARC, the PVN, dorsomedial hypothalamic nucleus (DMH), ventromedial hypothalamic nucleus (VMH) and lateral hypothalamus (LH) are the main regions that are responsible for the homeostatic control of energy balance. The ARC contains two distinct types of neurons: Neuropeptide Y (NPY) and agouti-gene related protein (AGRP) containing neurons and pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) containing neurons. NPY and AGRP are potent food intake stimulants while POMC and CART act as suppressors of feeding. These two populations of neurons interact reciprocally to control feeding [28]. Both types of neurons have afferent inputs from the brain stem (mainly the NTS), hypothalamic nuclei including PVN, DMH, and LH [29, 30] and are affected by signals from the periphery. POMC neurons produce alpha-melanocyte-stimulating hormone (α-MSH) which acts as an agonist at the melanocortin

receptor 4 (MC4) [31]. On the contrary, AGRP acts as the endogenous antagonist at the MC3 and MC4 receptors [32].

There is a number of peripherally produced signaling molecules that act both peripherally and within the CNS to influence food intake and energy expenditure. These molecules are either gut- or adipose tissue derived substances that act as hormones, providing the CNS with information about the energy balance status of the body and thereby affecting the central feeding nuclei [33](Figure 1.2). Ghrelin, for example, is an orexigenic substance (a substance which causes hunger and initiates feeding) secreted from oxyntic cells the stomach during fasting and its plasma level is greatly reduced in response to feeding [34]. Ghrelin is the endogenous ligand of the Growth-hormone secretagogue receptor (GHSR) which exerts its orexigenic action by acting on the hypothalamus (mainly at the ARC) through activation of NPY/AGRP neurons to stimulate food intake [35]. Peripherally, ghrelin causes stimulation of gastric acid and gastrin secretion as well as intestinal motility [36].

Along the GI tract, there are a number of anorectic factors that are secreted by different secretory cells and play important role in the regulation of feeding and metabolism. Intestinal L cells produce and release more than one peptide; namely PYY, GLP-1, and OXM. PYY is secreted in an inactive form and hydrolysed to a shorter active form; PYY (3-36). PYY (3-36) is released in proportion to the meal fat content [37]. It freely crosses the blood brain barrier (BBB) and acts mainly on the ARC especially on NPY neurons. PYY inhibits NPY neurons which send inhibitory inputs to POMC neurons, thus PYY releases this inhibition leading to its anorexigenic effect. In addition to the ARC,

there is some evidence showing the action of PYY on the brain stem (area postrema (AP) and NTS)[38]. GLP-1 and OXM are products from a single gene; the proglucagon gene. Systemic administration of GLP-1 increases the expression of c-Fos (an immediate early gene used as a marker of neuronal activation) in the AP and suppresses feeding, thus GLP-1 may act via activation of the brain stem [39]. GLP-1 acts peripherally to decrease gastric emptying thus increasing the feeling of satiety. GLP-1 has an incretin effect stimulating the secretion of insulin from the pancreatic β cells. OXM also acts as an inhibitor of food intake and stimulator of energy expenditure [11]. It has been shown to suppress feeding if administered centrally or peripherally [40]. Pretreatment with exendin (9-39); a GLP-1 receptor antagonist, results in the blockade of both GLP-1 and OXM-induced reduction of food intake which suggests that GLP-1 and OXM act via the same receptor. However, when exendin (9-39) is injected into the ARC, only the action of OXM is blocked suggesting that there might be distinct receptors mediating the effect of GLP-1 in the ARC [41]. Another gut–derived hormone important in the regulation of feeding behavior is cholecystokinin (CCK). CCK is secreted from intestinal I cells and was the first known anorexigenic gut hormone (a substance that induces satiety) [42]. CCK is secreted in response to feeding and is important for meal termination. The primary mechanism of action of CCK is through stimulation of afferent vagal nerves which activate the NTS leading to inhibition of feeding. CCK also acts peripherally to stimulate the pancreas to release digestive enzymes and the gall bladder to secrete bile [43]. From intestinal K cells, gastric-inhibitory peptide (GIP) is secreted and it also acts as an incretin [11].

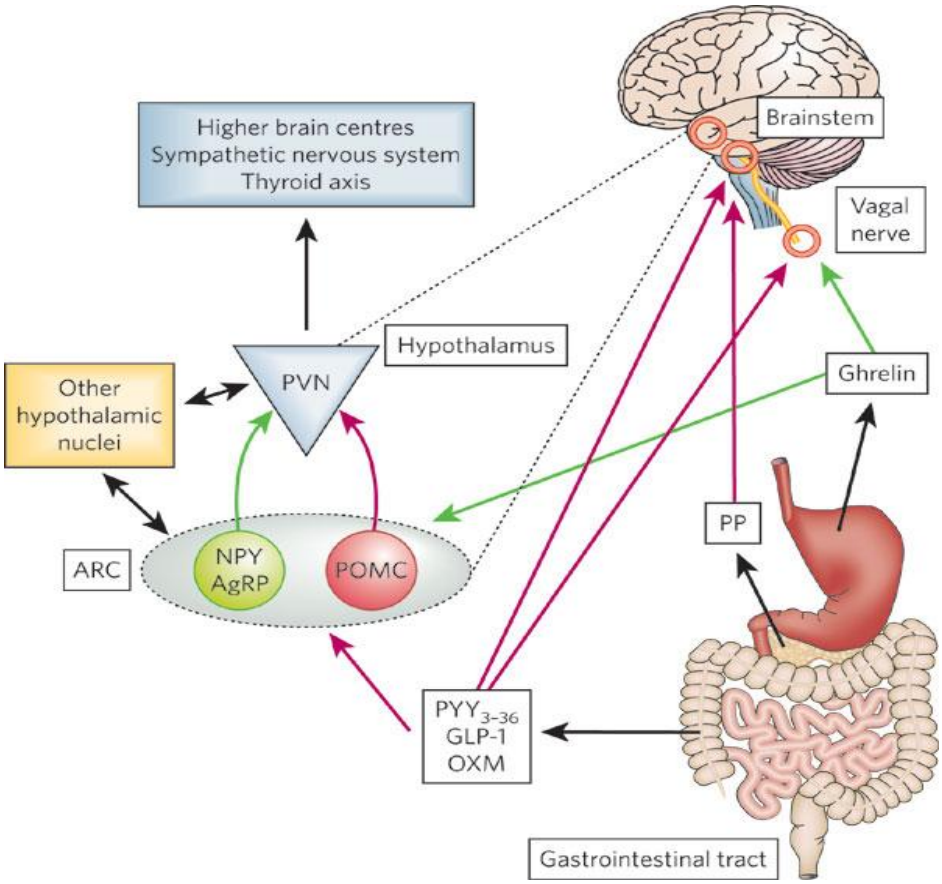

Figure 1.2: **The pathways by which gut hormones regulate energy homeostasis.** Green arrows indicate orexigenic signals and red arrows indicate anorectic signals. Reprinted by permission from Macmillan Publishers Ltd: Nature [11], copyright (2006, appendix B).

In addition to the gut secreted factors, adiposity-derived signals such as leptin play important role in the regulation of feeding and body weight. Leptin is produced from adipocytes and its circulating levels are proportional to body fat content. It acts peripherally to promote lipid oxidation and enhance insulin secretion [44]. Leptin crosses the BBB via binding to a short form of leptin receptor that acts as a carrier (Ob-Rb receptor). The main CNS targets for the action of leptin are found in the ARC, PVN

and LH as well as medial pre-optic areas [45]. Overexpression of the leptin gene in the ARC, PVN and LH leads to a reduction of both food intake and energy expenditure, however, overexpression in the median pre-optic area results in reduced energy expenditure only [46]. Leptin receptors are also present in the NTS [47] and peripheral administration of leptin causes neuronal activation within the NTS [48]. Besides leptin, adiponectin is also secreted from adipose tissue [49]. Plasma concentration of adiponectin increases after food restriction [50] as well as after weight loss [51]. Administration of adiponectin has been shown to reduce body weight by increasing oxygen consumption without affecting food intake [52].

Although receptors for the aforementioned signals were found to be expressed in the CNS, most peptides from the gut or periphery have limited or no access to the brain due to the presence of the BBB [53]. The BBB consists of the cerebral capillaries that lack fenestrations and transendothelial channels normally found in other blood vessels, thus preventing the transport of many circulating molecules into the brain [54]. Nevertheless, there are numerous alternate ways through which these molecules signal to the brain to control feeding behavior and energy homeostasis. For example,

- Signaling through the vagus nerve is a well established way to relay peripheral signals to the brainstem then to the hypothalamic feeding centers [55].
- The presence of specific saturable transporters, for example leptin brain transporters [56-58], that act as carriers to introduce circulating molecules into the brain.

- Transendothelial signaling in which circulating substances act on the endothelial side of BBB triggering the release of second messengers at the brain side [59].
- Through special brain regions that lack a fully functional BBB. These regions are called the circumventricular organs (CVOs) [60].

As the main focus of this thesis is the role of CVOs, a background on their role in homeostasis will be introduced in the following section.

1.3 The circumventricular organs (CVOs).

The CVOs, are specific brain regions localized around the third and the fourth cerebral ventricles. There are seven mammalian CVOs namely; the subfornical organ (SFO); the vascular organ of the lamina terminalis (OVLT), median eminence, pineal gland, subcommisural organ, the AP and the choroid plexuses of the lateral, third and fourth ventricles. Of these seven CVOs, only three contain neuronal cell bodies namely the SFO, OVLT and the AP [61] (Figure 1.3). Because these three CVOs are able to interact with circulating substances and have efferent neuronal connections to many brain regions, they were given the name “sensory CVOs” [62]. The rest of the CVOS are called secretory since they secrete hormones into the circulation.

The discovery of sensory CVOs goes back to the early 1900s, starting with the identification of the AP in 1906 [63], followed by the SFO in 1922 [64] and ending with the OVLT in 1926. Later on, the observation that the CVOs are stained with intravital dyes which do not cross into the brain, lead to the discovery that CVOs lacked a BBB [65, 66]. The importance of CVOs in physiological function began with the proposal of Borison and Brizze that the AP is the chemoreceptor trigger zone for vomiting [67]. In

1973, Simpson and Routtenberg showed that the SFO is the main site of action of circulating angiotensin II (AngII) to control drinking behavior [68].

The use of the expression of the early response gene; c-Fos as a marker of neuronal activity, significantly increased the knowledge and understanding of the role of sensory CVOs in different physiological functions [61]. More details on the use of c-Fos technique are discussed in Chapter 2.

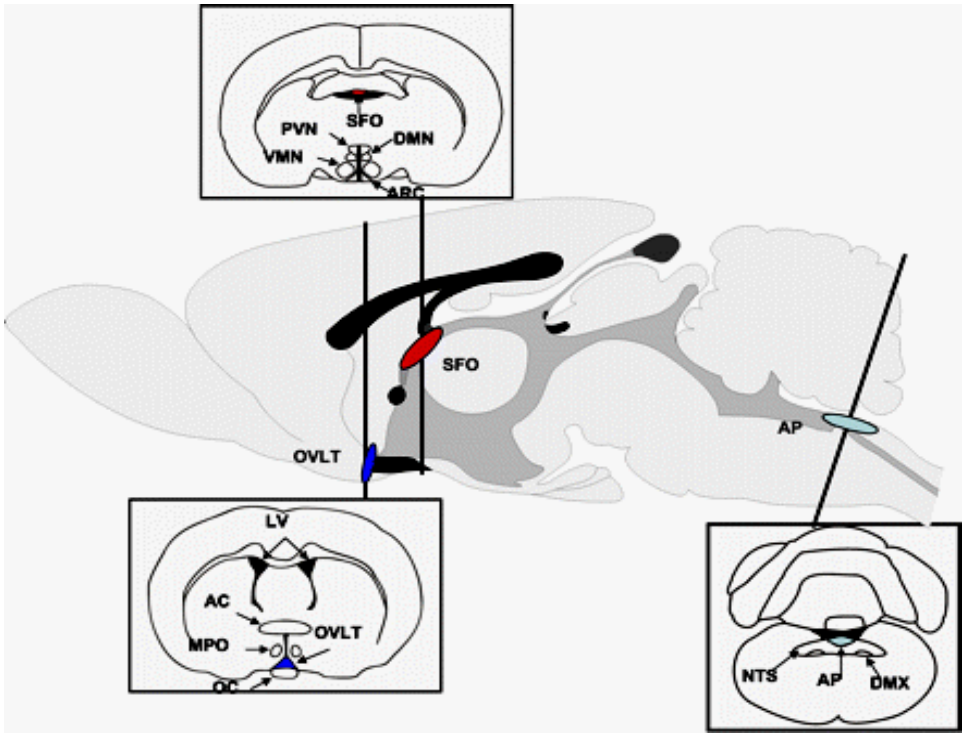

Figure 1.3: **A diagram showing sensory circumventricular organs.**

The center of this diagram shows a schematic representation of a sagittal cut through a rat brain illustrating the anatomical locations of all three sensory circumventricular organs. The three insets show coronal sections highlighting the positioning of the SFO and OVLT within the hypothalamus and the AP in the medulla. (used after permission from Fry *et al.*[**69**], appendix C)

CVOs are characterized by the presence of fenestrated capillaries and are highly vascularised. They are known to lack the complete BBB present at other brain regions. However, these unique characteristics of CVOs are not the only criteria that permit these brain regions to respond to circulating factors. The presence of a number of receptors for hormones, neurotransmitters and neuropeptides is another important factor contributing to the importance of CVOs in physiological functions [**60**, **62**, **70**, **71**]. The presence of these receptors has been confirmed by a variety of techniques such as receptor autoradiography, immunohistochemistry, electrophysiology and pharmacological studies [**61**]. Examples of neurotransmitter receptors include:

- muscarinic cholinergic receptor (M1) present in the SFO [72] as well as OVLT and the AP [61].
- Receptors for the γ-aminobutyric acid (GABA) which is the main inhibitory neurotransmitter in the SFO [73]. Glycine (which is another inhibitory neurotransmitter) receptors have been found in both the AP and the SFO [73, 74].
- Different subtypes of serotonin receptors are also found in the SFO [75] as well as in the AP [76].
- Angiotensin receptor type 1 (AT1) is also found to be expressed in high densities in all the three sensory CVOs [77, 78].

In addition to expression of receptors, neurotransmitters have also been found to be present in sensory CVOs for examples acetylcholine, catecholamines, serotonin, AngII, GABA and vasopressin [61].

1.3.1 Role of CVOs in homeostatic functions.

The sensory CVOs have essential roles in the control of metabolic and endocrine functions. The use of c-Fos as a marker of neuronal activation has shown the involvement of CVOs in different physiological functions. For example, sodium depletion or dehydration caused increased c-Fos expression in the NTS and SFO [79]. AngII, which plays an important role in blood pressure and fluid homeostasis, increased c-Fos expression in the SFO and OVLT, thereby emphasizing the important role these CVOs have in water and electrolyte balance [80]. The AP also has shown increased c-Fos expression in response to emetic substances and elevation in blood pressure [81, 82]. The actions of vasopressin as well as AngII as modulators of drinking behavior and cardiovascular function are also found to be mediated by the sensory CVOs [83, 84]. Receptors for relaxin; a hormone secreted from the corpus luteum during pregnancy, has been identified in the SFO and OVLT[85]. Intravenously administered relaxin caused an increase of c-Fos immunoreactivity in the SFO and OVLT and electrophysiological studies have shown that relaxin stimulates SFO neurons in a brain slice preparation [86].

The sensory CVOs have also been implicated in immune function. Peripheral administration of interleukin-1β (IL–1β), tumor necrosis factor-α (TNF-α) and lipopolysaccaride (LPS), results in increased c-Fos expression in the OVLT [87-90]. The SFO has also been identified as a target for the actions of IL-6, IL-1 β and TNF-α [91–93]. An interesting observation which suggests the integrative roles of CVOs was reported by the work of Plata-Salaman and colleagues [94, 95]. They found that intracerebroventricular administration of IL-1 β results in reduction of food intake in a

similar manner to those observed in response to the pancreatic hormone amylin [94, 95]. Using *in situ* hybridization for localization of the mRNA expression of the inhibitory factor kappaβalpha (a marker for immune response), it was found the AP also plays a role in the early response to intraperitoneal LPS administration [96]. In addition to immunohistochemical techniques, electrophysiological studies showed that the SFO neurons depolarize in response to IL-1β [97] while TNF-α increases the firing rate of the OVLT cells [98].

Lesion studies have also provided considerable evidence to suggest the importance of CVOs in different physiological functions. For example, dual lesion of the SFO and the AP in rats resulted in a sustained and significant decrease in resting blood pressure and heart rate [99] as well as decreased food intake and body weight [100] . In addition, combined SFO and OVLT lesions increased water and saline drinking on a daily basis [101]. The OVLT plays a role in reproduction and gonatrophic hormone secretion. In female rats, lesion of the OVLT resulted in elevated luteinizing hormone releasing hormone content of the mid-basal hypothalamus and in serum prolactin levels and reduced serum levels of luteinizing hormone and follicle stimulating hormone [102]. Ablation of SFO results in disrupted estrous cycle prolonged diestrous phase [103] and this was also observed with OVLT lesions [104].

The aforementioned literature represents some examples showing the involvement of sensory CVOs in different homeostatic functions from cardiovascular control to reproductive functions. As the work of this thesis focuses on the role of CVOs in the function of hormones controlling feeding and energy balance, the next section

will provide more detailed review on the current knowledge about the role of CVOs in this homeostatic function.

1.3.2 Role of CVOs in feeding and metabolism.

Of the three sensory CVOs, the AP and the SFO are the main CVOs which have relevant data supporting their role in the regulation of feeding and energy balance. Figure 1.4 shows examples of GI hormones involved in the control of food intake and energy balance which have been found to be acting on the AP and the SFO using receptor localization or by pharmacological evidence. Evidence for the involvement of each CVO in metabolism and energy balance behavior will be discussed in details below.

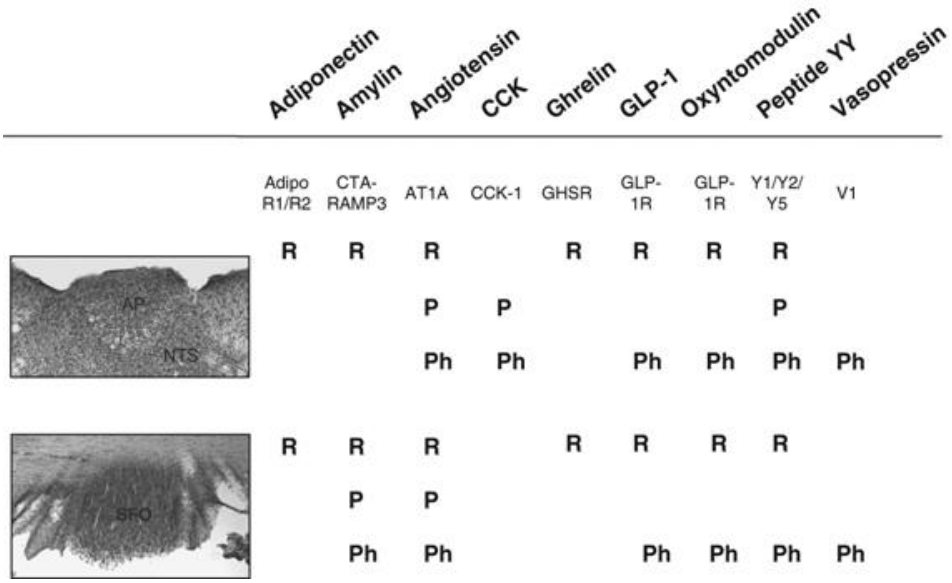

Figure 1.4: A figure summarizing some gastrointestinal hormones involved in energy homeostasis in the AP and SFO.

R: mRNA localization of the peptide receptor, P: evidence for the presence of the peptide and Ph: pharmacological evidence using specific agonists or antagonists. (used after permission from Hoyda *et al.*[105], appendix D)

1.3.2.1 Role of the area postrema in energy balance.

The AP has a well-established role in the regulation of energy balance. The AP have been shown to express receptors for numerous peripheral signals involved in feeding and metabolism [106] and electrophysiological studies have shown that the excitability of AP neurons is affected by a number of signals involved in metabolic control [39, 107-109].

Evidence of the involvement of the AP in regulation of feeding comes from studies measuring c-Fos induction in response to various aspects of feeding. For example, it was shown that feeding rats with a satiating meal lead to increased c-Fos in the AP [110] and Johnstone *et al.* were able to replicate this effect in response to 2-hour feeding regimen [111]. Fraser and Davison also studied meal induced activation of AP and they found that the induction of c-Fos in this area is not dependent on CCK receptors [112]. A robust increase in c-Fos immunoreactivity was observed in response to acute gastric distension induced by intake of a large volume of liquid diet following a 22 hour fasting [113]. Duodenal infusions of glucose, linoleic or amino acids [114, 115] as well as a lipid emulsion [116] have been shown to induce c-Fos in the AP. Moreover, Emond *et al.* showed that prior experience with gastric infusions did not affect the number of c-Fos positive neurons within the AP. They also studied the effect of the flow from the stomach to the intestine and combined gastric load and duodenal nutrient and found that combination of both infusions result in higher c-Fos expression levels than either gastric or duodenal infusions alone [117].

Besides measuring c-Fos immunoreactivity, a lesion study showed that rats subjected to AP lesion ate larger meals than control rats, suggesting the importance of AP in the feedback signals controlling food intake [118]. However, it should be noted that in a series of experiments, Edwards and Ritter reported that AP-lesioned rats did not show difference in food intake compared to controls, but showed an increased consumption of flavoured food suggesting that ablation of AP may be involved in the enhancement of the quality of preferred flavoured food [119].They also reported that vagotomy did not completely abolish AP-lesion induced overingestion of palatable food [120]. In another study performed by the same researchers, they concluded that lesion of the AP caused overconsumption of palatable food without affecting the animals' ability to control total caloric intake [121]. The apparent discrepancy in the effect of AP lesions might be due to the difference in the method for lesion; Stricker *et al.* used physical aspiration of the AP region while Edwards and Ritter used thermal induced lesion. Moreover, Stricker *et al.* were monitoring food intake electronically and more frequently than Edwards and Ritter who measured daily food intake manually.

In addition to food intake, a number of GI and adiposity-derived signals have been studied for their effect on the AP. The anorectic peptide amylin, which is secreted from the pancreas during feeding [122] has been shown to have excitatory actions on 50% of tested AP neurons and 50% of the amylin-sensitive neurons were found to be responsive to high glucose concentrations [123]. These data provided evidence that during feeding, when circulating glucose and amylin are increased to their maximal, neurons of the AP will be strongly activated [124]. *In vivo*, pretreatment with amylin

receptor antagonists results in inhibition of feeding-induced c-Fos expression in the AP as well as a reduction of amylin-induced inhibition of feeding [125]. Moreover, microinjection of an amylin receptor antagonist alone into the AP results in increased food intake [126]. Lesion studies also provided evidence of the importance of the AP in amylin-induced action. Lesion of the AP resulted in blockade of amylin-induced inhibition of food intake [127] as well as c-Fos expression in the brain stem [125].

CCK, a critical peptide hormone implicated in the termination of feeding, acts also through activation of the AP. Similar to amylin, electrophysiological studies showed that neurons of the AP respond to CCK in slice preparations and likewise, some CCK-sensitive neurons are glucose-sensitive [109]. c-Fos studies provided evidence for the action of circulating CCK on the AP. Systemic CCK administration results in increased c-Fos expression in both the AP and the NTS, and this effect is attenuated by either the absence of CCK receptor 1 subtype ($CCKR_1$) [128] or by prior treatment with a $CCKR_1$ antagonist [129]. Hayes and Covasa found that gastric distension enhances CCK-induced c-Fos expression in the AP via activation of the serotonin receptor subtype; $5\text{-}HT_3$ [130]. Reduction of CCK-mediated inhibition of feeding was observed in animals with AP-lesion emphasising the role AP plays in mediating CCK-effect on food intake [131]. On the other hand, Edwards *et al.* demonstrated that the AP was not essential for CCK-induced reduction of food intake [132].

The AP has also been implicated in the actions of other anorexegenic hormones that are also secreted from the GI tract such as GLP-1, OXM and PYY. Systemic administrations of GLP-1 receptor agonist [39] or OXM [133] as well as PYY [134] result

in increased c-Fos expression in the AP. Interestingly, lesion of the AP did not affect PYY induced reduction of feeding but instead enhanced its effect [135]. This can be explained by the results showing electrophysiological actions of PYY on membrane excitability of cultured AP neurons. PYY1-36 was found to have depolarizing (excitatory) while PYY3-36 induced hyperpolarizing (inhibitory) actions [69]. Moreover, ablation of the AP blocked the ability of PYY to inhibit CCK-induced pancreatic secretion [136].

As mentioned earlier in this chapter, ghrelin is the only peripheral orexegenic hormone secreted from the stomach. Neuronal activation within the AP has been observed in response to ghrelin; *in vivo* using c-Fos [137] as well as *in vitro* by electrophysiology [107].

Leptin and adiponectin are two adipose-derived signals that act on the AP. Direct application of adiponectin to dissociated AP neurons results in hyperpolarizing and depolarizing responses [108]. While there is no available data on the direct effect of leptin in AP neurons, leptin receptors are found to be expressed in the AP [138] suggesting a role of AP in the actions of leptin. Expression of p-STAT (phosphorylated-signal transducer and activator of transcription 3) signalling in the AP in response to systemic leptin administration provides further evidence of the involvement of AP in leptin mediated effects [139].

All the above literature shows that the AP is not just a brain site through which blood-borne signals are transferred into the CNS, but has the potential to play an integrative role in controlling the outputs of multiple signals.

1.3.2.2 Role of the subfornical organ in energy balance.

Unlike the AP, the SFO has not been extensively studied in terms of its effect on food intake and energy balance. However, the SFO has a potential role in energy balance supported by the expression of different receptors for signals involved in controlling energy homeostasis as shown by a microarray study of the transcriptome of the SFO tissue [140]. Another factor that suggests the potential role of the SFO in controlling metabolic functions is its neural projections to hypothalamic areas with well studied roles in energy homeostasis (Figure 1.5). Efferent projections from the SFO have been identified by anatomical studies showing projections to the ARC using retrograde tracing [141] and LH [142] and PVN [143] using anterograde tracing.

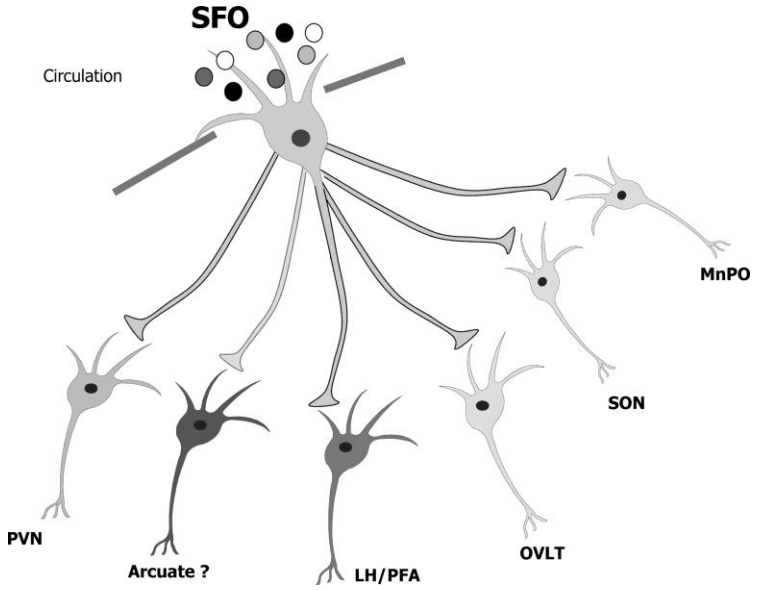

Figure 1.5: **Schematic presentation of SFO efferent projections to important autonomic nuclei.**

Obtained from Smith and Ferguson [144]; copyright permission was not required.

Increased c-Fos expression in the SFO was observed after systemic ghrelin administration [137]. Intracerebroventricular (icv) injections of ghrelin increased food intake and inhibited water intake [145, 146], effects which are similar to the action of systemic ghrelin [147]. While ghrelin is primarily known for its orexegenic effect, its action on the SFO might be mediating another effect supporting a role for the SFO as an integrator of circulating peptides that control feeding. Nesfatin-1, a peptide that has been suggested to have a satiety action [148], has been shown to induce c-Fos expression on the SFO after icv injection [149]. Nesfatin-1 also causes changes in membrane potential of dissociated SFO neurons [150]. Lesion of the SFO resulted in reduced c-fos mRNA in specific brain structures in response to albumin conjugated PYY (a form of PYY that does not cross the BBB) [151]. However, in this study, only combined SFO/AP lesions resulted a reduction of PYY induced reduction of food intake. In a study by Baraboi *et al.*, they showed that lesion of the SFO and the AP resulted in blunted c-fos mRNA in specific brain area in response to a GLP-1 receptor agonist without affecting GLP-1 induced satiety effect [100].

Leptin induced STAT3 signaling has been observed in the SFO in response to leptin administration [152]. In the same study, the presence of leptin receptor in the SFO was confirmed using immunohistochemical localization as well as the presence of mRNA for the leptin receptor. Dissociated SFO neurons responded to bath application of leptin confirming the action of leptin on the SFO [152]. Leptin-induced effect on the SFO was studied by direct microinjection of leptin into the SFO which resulted in a rapid decrease in blood pressure [153]. Interestingly, in this study, the leptin-induced

cardiovascular effects were abolished in a model of high fat diet induced obesity [153]. In a study by Young and colleagues, deletion of leptin (ObR) receptor was achieved by SFO-targeted microinjections of an adenovirus encoding Cre-recombinase in $ObR^{flox/flox}$ mice. They found that selective ablation of ObR in the SFO did not influence the decreases in either food intake or body weight in response to daily systemic or icv administration of leptin. However, leptin-induced renal sympathetic excitation was abolished in mice with SFO-targeted ablation of ObR [154].

A functional role for the SFO in the regulation of energy status has been supported by electrophysiological studies. For example, adiponectin has been shown to affect the excitability of dissociated SFO neurons and the presence of adiponectin receptors was confirmed by real time PCR [155]. Apelin, another adipocyte-derived signal [156] with effects on food intake [157], was also found to induce electrophysiological changes in the SFO neurons and when microinjected into the SFO a reduction of blood pressure is observed [158]. Pulman *et al.* observed that populations of SFO neurons respond either to amylin or ghrelin which suggested that orexigens and anorexigens both acted at the SFO, but via different neuronal pathways [159]. Moreover, a study by Smith *et al.* showed that acute, short duration SFO stimulation induced feeding in satiated rats [160]. In addition to these metabolic signals, SFO neurons has been found to be glucose sensitive permitting them to monitor the levels of circulating glucose [161].

All these data suggests the important role of the sensory CVOs in modulating metabolic function. The AP and the SFO do not just act as sites of action of circulating

signals but they have an integrative role sensing and modifying the CNS response to various energy balance related signals.

1.4 General Hypothesis and Aims.

There is growing evidence suggesting that the consumption of a high fat diet leads to adaptive changes within the GI tract. For example, increased expression of mucosal enzymes and an overall increase in intestinal mass in rats were reported after long term intake of high fat diet [162]. Also, adaptation to high fat diet was accompanied by attenuated brainstem and enteric neuronal c-Fos expression in response to intestinal fat content which may contribute to increased body weight and food intake in animals chronically exposed to high fat diet [163].

These observed peripheral changes increases the probability that parallel changes within the brain feeding and energy balance centres take place. One possible way for these central changes to occur is through changes in the pattern of interaction between the brain and the gut. It has been shown that rats fed a high-fat diet overexpress hypothalamic orexin-A and NPY, both of which are orexigenic mediators, leading to higher food intake [164]. Another study in mice showed that chronic intake of a high fat diet lead to the disruption of hypothalamic leptin signaling, which helps to explain the increased body weight and obesity in response to high fat feeding [165]. However, most of the existing literature has focused on studying the adaptive changes within the hypothalamic nuclei. The changes in the response of circumventricular organs to circulating signals in normal versus high-fat diet feeding are unclear. Therefore, I hypothesize that adaptation in the responsiveness of the sensory CVOs to gut-derived

signals underlies part of the central plasticity which results in an altered defense of higher body weights and the development of obesity.

I will test the hypothesis that the development of obesity in response to a high fat diet alters the responsiveness of the CVOs to the gut-derived signal CCK.

As mentioned in the introduction, sensory CVOs represent potential targets for circulating factors involved in the regulation of feeding and energy balance. One of these factors is the gut hormone CCK which is a short term suppressor of feeding with proposed effects on long term energy balance and body weight. Evidence of CCK's involvement in regulation of body weight is the observed phenotype of the Otsuka Long Evans Tokushima Fatty (OLETF) rats, which lack the expression of CCK_1R and are characterized by increased meal size with subsequent development of obesity [166].

CCK-8 does not readily cross the BBB [167] and its receptors have been found to be expressed in the AP and the SFO making these regions potential target for its action. Accordingly, the specific aims of my thesis are as follows:

Aim 1: To study the pattern of activity of CVOs neurons in response to CCK (Chapters 2 and 3).

This aim will be addressed by assessing of neuronal activity in sensory CVOs (namely SFO, AP and OVLT) in response to peripheral CCK administration (using a dose of CCK that inhibits food intake). I will use c-Fos immunoreactivity as a marker of neuronal activity. In addition to c-Fos, I will assess neuronal activity by another technique by assessing neuronal immunoreactivity of phosphorylated extracellular signal regulated kinase 1 and 2 (pERK1/2) in response to CCK. pERK is the active form of

ERK enzyme that has been established to be one of key signals in the CCK transmembrane signalling cascade important for its inhibitory effect on food intake [168]. This approach has previously been used to study CCK-mediated activation of the NTS [168, 169].

In order to define the CCK receptor subtype mediating the effect of CCK on SFO, measurement of CVO activity in response to CCK in animals pre-treated with either specific CCK_1 or CCK_2 receptor antagonists (alone and in combination with CCK) will be performed. These experiments will follow the initial behavioral experiments investigating the effects of each CCK antagonists on CCK-induced reduction of food intake.

To study the contribution of the vagus nerve, as an important mediator in CCK induced reduction in food intake, rats subjected to subdiaphragmatic vagotomy will be used to study the effect of CCK. The effect of endogenous CCK released in response to a meal will be also investigated and compared to the effect of exogenous CCK.

Aim 2: To study the effect of a high-fat diet on the pattern and extent of CVOs activation in response to CCK (Chapter 4).

Alterations of CCK action have been reported in high fat-diet induced obesity. For example, rats consuming HFD have been shown to have modified physiological actions of CCK such as reduction of the effect of i.p. CCK on gastric emptying [170], brainstem neuronal activation [163], and food intake [171]. Therefore, understanding any modified responsiveness of the sensory CVOs in diet-induced obese rats is critical for understanding the potential roles of the CVOs in the development of obesity.

CCK-induced activation of c-Fos in the CVOs will be evaluated in rats fed high fat diet. I will use rats fed high-fat diet that become obese and those that remain lean will be used as controls. In order to control for the diet itself, I will compare responses to chow-fed-age-matched rats.

Chapter 2 Effect of CCK on the activity of sensory CVOs.

2.1 Introduction

2.1.1 Cholecystokinin

As mentioned earlier in Chapter 1, cholecystokinin is one of the gut hormones that is released in response to feeding and is responsible for meal termination. In 1928, CCK was demonstrated to induce contraction of the gall bladder [172]. This was followed by its purification, chemical characterization and the localization of CCK's site of secretion to be the intestinal (I) cells [173]. In 1973, Gibbs and colleagues reported that the systemic administration of CCK caused a reduction in food intake in rats [42]. CCK acts on two distinct receptors, namely; $CCKR_1$ and $CCKR_2$. Both receptors belong to the seven transmembrane G-protein coupled receptors family. CCK_1 receptors are mainly distributed peripherally with some central distribution, while CCK_2 receptors show a largely central distribution, but there are also the peripheral CCK_2/gastrin receptors which are localized on parietal cells and GI smooth muscle cells [174].

A number of signaling pathways are activated in response to the activation of both types of CCK receptors (Figure 2.1). The Gq member of the G-protein receptor family is coupled to both CCK1 and CCK2 receptors [175]. The principal target of activation of Gq coupled receptors is phopolipase C (PLC) which result in the formation of inositol triphosphate (IP3) leading to the release of calcium from the intracellular stores. In addition to activation of PLC, phospholipase A2 and phospholipase D (PLD) are activated by CCK receptors. Activated PLD leads to the formation of diacylglycerol and activation of protein kinase C (PKC). PKC subsequently activates the mitogen activated

protein kinase (MAPK) pathways with subsequent activation of the ERK1/2 pathway [174]. Moreover, the involvement of other signaling pathways have been reported including Gs coupled CCK1 receptor with subsequent activation of adenylate cyclase and production of cyclic AMP [176], increased nitric oxide production [177], non-PKC dependent MAPK activation and CCK2 mediated activation of JAK/STAT pathway (Janus Kinase/ Signal Transducers and Activators of Transcription) [178].

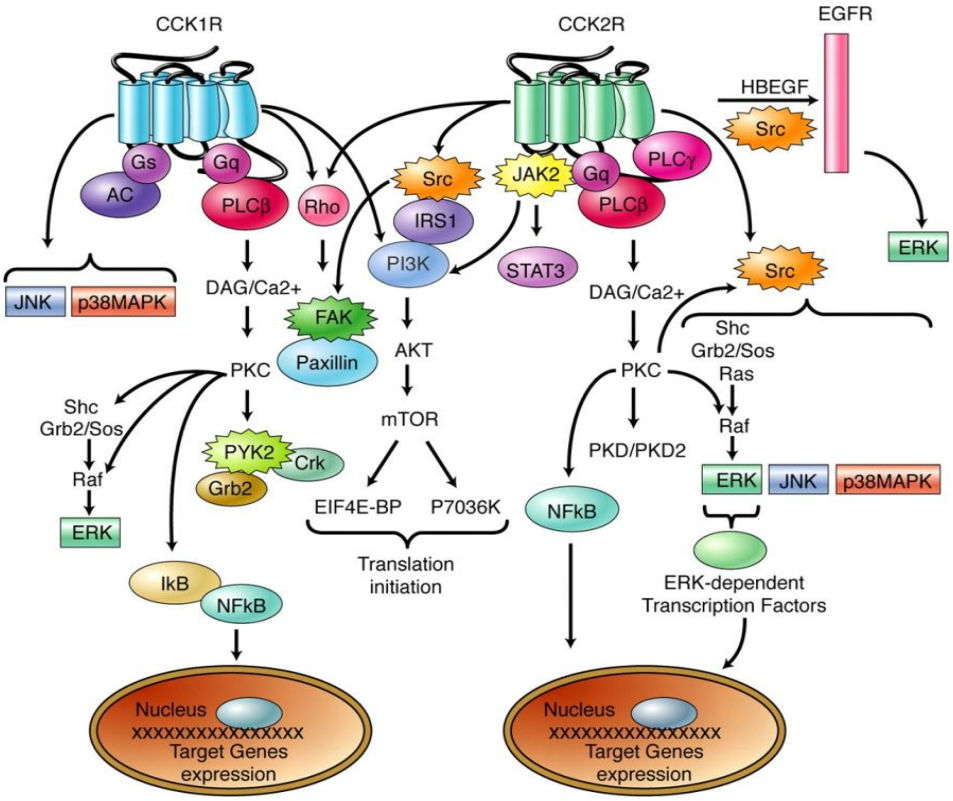

Figure 2.1: Schematic description of the signaling pathways activated by CCKR$_1$ and CCKR$_2$.

Used from Dufresne *et al.* [174], copyright permission is not required.

CCK is present in different molecular forms. It is initially synthesized as 115-amino acid pre-procholecystokinin [179] This form is then subjected to extensive post-translational modifications. A number of prohormone convertases are thought to be involved in the conversion of the 115 amino acid form molecules into various forms of CCK with different sizes [180]. Consequently, various forms of CCK exist in different parts of the body and in the circulation. Longer forms of CCK (such as CCK-22 and CCK-33) are mostly found in the gut and the plasma, whereas, the shorter sulphated c-terminal form (CCK-8S) is the most abundant form in the brain [181] (Figure 2.2). Although a study by Reeve *et al.* [182] has shown that CCK-58, the full length CCK active form, is the only detectable form of CCK in the rat circulation, the sulphated form of CCK-8 is the most commonly used form in feeding and behavioral studies, because it is the commercially available stable form of CCK. This molecular form has been shown to have similar affinity to CCK_1 receptor compared to the longer forms. In addition to binding to $CCKR_1$, CCK-8S binds to $CCKR_2$ with comparable affinity [174].

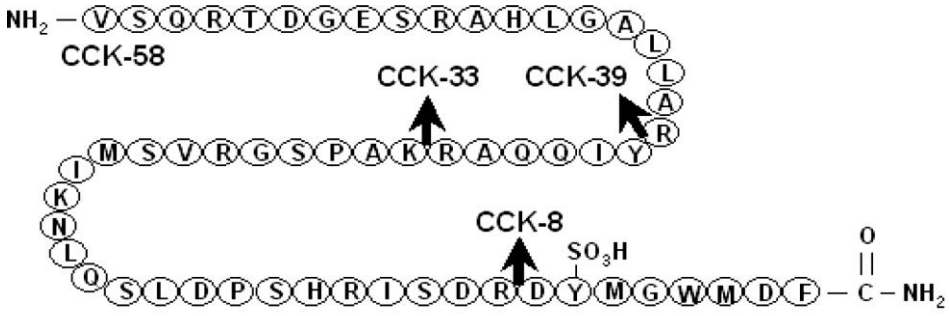

Figure 2.2: Primary structure of cholecystokinin with illustration of some possible sites for cleavage into different forms.

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Sensory vagal afferents are known as the main site of CCK action for reducing food intake. While extensive evidence exists to suggest that vagal afferents are essential for the CCK-dependent inhibition of food intake [183-187] , the vagus might not be the only site of action of CCK [188-190] . For example, it has been shown by many groups that either chemical or surgical ablation of the vagus attenuated CCK inhibition of food intake in rats. For example, chemical ablation of the afferent sensory vagal nerves was performed mainly by the use the neurotoxin capsaicin where either its application into the fourth ventricle (to ablate central vagal afferents) [183, 184] or by intraperitoneal injection (to affect peripheral vagal afferents) [184, 187] attenuated the effect of CCK on food intake. In addition, surgical ablation of the vagus by subdiaphragmatic vagotomy has been shown to reduce the effect of CCK on food intake [185, 186]. On the other hand, other experiments suggested the presence of additional pathways for CCK action. For example, Reidelberger demonstrated that blockade of the CCK_1 receptors via the CCK_1 antagonist devazepide induced an increase in food intake in vagotomized rats suggesting that CCK inhibits food intake by other mechanisms independent on an intact vagus which may include central sites or other non vagal peripheral sites [190]. In a similar experiment, capsaicin was utilized to ablate vagal afferents in rats and devazepide was shown to maintain its food intake-increasing effect [189]. This might be as a result of the action of devazepide on non vagal peripheral $CCKR_1$ sites or due to the central action of devazepide which cross the blood brain barrier. More recently, Zhang and Ritter [188] reported that reduction of food intake by CCK was abolished in vagotomized and capsaicin-treated rats only if CCK is given intraperitoneally, while the

effect of intravenous CCK was only partially attenuated in these rats, raising the possibility that circulating CCK may access extra-vagal sites of action.

In addition to the actions of CCK on the vagal afferents, CCK plays an important role in controlling the function and neurochemical phenotype of vagal afferents neuronal cell bodies that forms the nodose ganglion [191]. Feeding induces an increase in nodose ganglion expression of Y_2 receptors (receptors for the gut hormone PYY) and a reduction in the expression of CB_1 (endocannabinoid receptor) and this effect is mainly mediated by CCK [192, 193]. In addition to changes in receptor expression, CCK also induces changes in expression of neuropeptide transmitters within the nodose neurons such as increased CART expression [194] and reduced MCH [195] in response to CCK . These receptors and neurotransmitters are important players in controlling feeding and energy balance (previously discussed in Chapter 1).

Although CCK is well-known as a short term suppressor of feeding, evidence for possible effects on long term energy balance and body weight is continuously emerging in the literature. For example, lack of the CCK_1 receptor in Otsuka Long Evans Tokushima Fatty (OLETF) rats, leads to a phenotype that is characterized by increased meal size (due to absence of a CCK meal terminating effect) with subsequent development of obesity [166]. This suggests that CCK is a possible contributor to body weight regulation. Moreover, interactions between CCK and other energy balance signals have been shown where CCK exerts a synergistic effect with these signals. One example of such interactions is the interaction between CCK and the adiposity-derived energy balance hormone: leptin. A study has shown that long term co-administration of

CCK and leptin caused a significant reduction of daily food intake and body weight in rats compared to leptin treatment alone [196], emphasizing the role that CCK can have for energy balance regulation.

2.1.2 Hypothesis and Aim.

Based on the fact that CCK-8 does not readily cross the blood brain barrier [167] and on the aforementioned literature that suggests the presence of extra vagal site of actions of CCK which may or may not be involved in the effects of CCK on food intake and according to my preliminary experiment that showed response of the SFO and the AP only to systemic CCK, I hypothesized that in addition to the vagal pathway, CCK acts by activating the sensory circumventricular organs; AP and the SFO which lack the presence of a complete blood brain barrier.

The expression of the immediate early gene c-Fos has been extensively used as a marker of neuronal activation in response to a stimulus [197]. It has been also used to map neuronal activity in response to CCK [198-200]. In addition to c-Fos, increased levels of the phophorylated form of ERK1/2 (which is a step in the MAPK signaling pathway involved in $CCKR_1$ and $CCKR_2$ signaling) has been demonstrated in response to CCK [169, 201]. Accordingly, the aim of this Chapter is to identify using c-Fos and p-ERK immunoreactivities, other sites of action of CCK- if any - and to determine the receptor subtype mediating this effect.

2.2 Materials and Methods.

2.2.1 Animals.

Experimental procedures were carried out in accordance with the guidelines of the Canadian Council on Animal Care after approval from the Health Sciences Animal Care Committee of the University of Calgary. Male Sprague-Dawley rats between 120-150 g were purchased from Charles River (Montreal, Quebec, Canada). Upon arrival, they were housed under specific pathogen free conditions on a reversed 12-hour light-dark cycle with free access to food and water for one week before the experiment.

2.2.2 Compounds.

Cholecystokinin (sulphated CCK-8; catalog no. 069-03) was purchased from Phoenix® (Phoenix Pharmaceuticals, Burlingame, CA, USA). Devazepide and L-365,260 were purchased from Tocris® (Tocris Bioscience, Minneapolis, MN, USA). CCK-8 solution was prepared (according to manufacturer's instructions) by dissolving 50 microgram of CCK in 200 μl of 0.2 M ammonium bicarbonate solution then completing the volume with sterile saline solution. Antagonist solutions were prepared by dissolving the powders in DMSO, Tween 20 and sterile saline solution in a ratio of 1:1:8.

2.2.3 Feeding studies.

In order to optimize the experimental conditions for the effect of CCK on food intake, feeding studies were performed to determine the effect of CCK-8 on food intake before performing c-Fos and p-ERK studies.

To determine the dose of CCK-8 to be used, sucrose intake was measured at the beginning of the dark cycle in response to three doses of CCK-8; 2, 8 and 16 μg/kg. Prior

to the experiment, 15% sucrose intake was recorded daily for 30 minutes until a stable baseline was reached. On the day of the experiment 30 minutes before the dark cycle, 18 hours fasted singly housed rats (n=16) were randomly divided into four groups receiving vehicle or one of the three doses of CCK-8. Five minutes after the intraperitoneal (i.p.) injection, the 15% sucrose solution was offered to each rat and the amount of sucrose consumed at 30 minutes was recorded.

The same procedure was then repeated using the most effective dose but with standard rat chow pellets instead of sucrose. Fasted rats were assigned to two groups, one group received i.p. CCK-8 (16 μg/kg) and the other served as a control receiving vehicle. Five minutes after injection, pre-weighed amounts of rat chow pellets were offered to each rat and the amount of food consumed at 30 and 60 minutes was recorded. Similar experiments were performed to choose the appropriate dose of the CCK receptor antagonists; devazepide and L-365.260. Three doses of each antagonist were tested for blocking the effect of CCK-8 on food intake, 1 mg, 600 μg and 300 μg for devazepide and 500μg, 200 μg and 100 μg for L-365.260. Antagonists were injected i.p. 30 min prior to the injection of CCK-8. Pre-weighed food pellets were presented 5 min after the i.p. injection of CCK-8 and food intake was measured by measuring the amount left at 30 and 60 minutes after injection.

2.2.4 Determination of plasma CCK concentrations.

Rats that were fasted overnight were divided into two groups (n=4 each). Blood was withdrawn from the tail into EDTA coated tubes from each animal at time 0. At the beginning of the dark cycle, one group received an intraperitoneal injection of CCK-8

(16μg/kg) and the other group was provided with a liquid Ensure® meal (15ml, which is fully consumed in 20min). Blood was withdrawn at 30 and 60 min after injection or after the start of feeding. Collected blood was centrifuged at 3,000 rpm for 15 min at 4^0C. The plasma samples were stored for a limited time at -80^0C before plasma CCK was measured using an ELISA kit (Catalog number EK-069-04, Phoenix, Belmont, CA) following the manufacturer's instructions as outlined in previous literature [202-205].

2.2.5 c-Fos immunohistochemistry.

Rats that were adapted to daily handling and injection with saline for at least 5 days were fasted overnight. On the day of the experiment, rats were treated with CCK-8 (16 μg/kg, i.p.), with or without CCK receptor antagonists or their respective vehicles one hour before the dark cycle. CCK antagonists were injected 30min prior to the injection of CCK. Ninety minutes after CCK injection, animals were anesthetized with sodium pentobarbital (50 mg/kg), perfused intracardially with 200 ml saline followed by 300 ml 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.3). Brains were carefully removed and fixed in 4% paraformaldehyde at 4°C overnight, and then cryoprotected with 20% sucrose in PBS at 4°C overnight. Specimens were embedded in OCT compound (Tissue-Tek, Sakura Finetek Japan Co., Ltd., Tokyo, Japan) and were sectioned in the coronal plane on a cryostat (35 μm). Floating sections were collected with reference to the rat brain atlas [206] containing the subfornical organ (SFO, bregma -0.58 mm), nucleus of the solitary tract (NTS, bregma -14.04 mm) and area postrema (AP, bregma -14.04 mm).

Floating sections were washed three times in 10 minute intervals in PBS containing 0.1% Triton X-100 and then incubated in blocking buffer comprised of PBS containing 10% normal donkey serum for 1 hour at room temperature. Sections were incubated in primary antibody rabbit anti c-Fos (1:2000; Oncogene Science, Cambridge, MA, USA) at 4 °C for 48 hours. Tissues were then washed in PBS containing 0.1 % Triton X-100 3 times for 10 min and incubated in donkey anti-rabbit CY3 (1:100; #711-166-152, Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) at room temperature for 2 h. Sections were mounted and examined using a Zeiss Axioplan fluorescence microscope (Carl Zeiss, Jena, Germany). The gray-scale images were captured by digital camera (Qimaging, Surrey, BC, Canada). Numbers of c-Fos immunoreactive neuronal nuclei in SFO, AP and NTS were counted using a computer-based image analysis system (ImagePro plus software) by defining both a minimum intensity value and size by an observer unaware of treatment conditions. The areas of each region was delineated according to landmarks in the brain atlas Paxinos and Watson [206] as shown in Figure 2.3. The mean number of c-Fos-immunoreactive cells from 3 sections per animal was compared among different animal groups and images for the same region were obtained under the same shutter time.

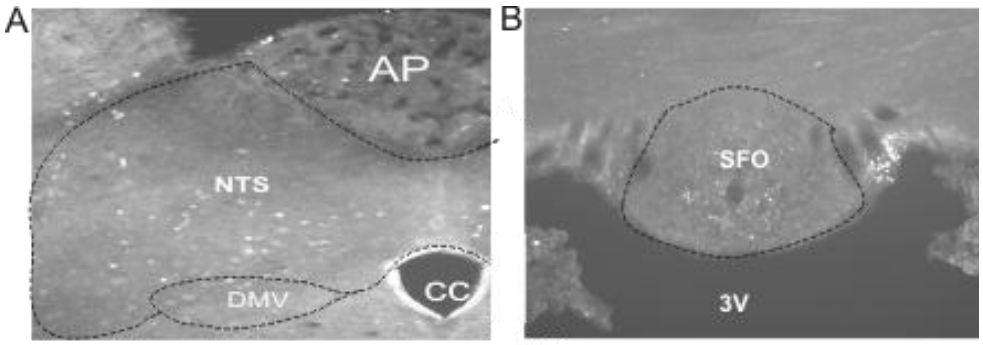


Figure 2.3: Schematic diagram defining the regional borders of AP, NTS and SFO used for c-Fos counting.

AP: area postrema, NTS: solitary tract nucleus, DMV: dorsal motor vagus, cc: central canal, SFO: subfornical organ and 3V: third ventricle

2.2.6 p-ERK immunohistochemistry

Immunoreactivity for p-ERK was assessed using the above procedure modified to collect the brain tissues 15 minutes after CCK-8 (16 μg/kg) administration, and with the primary antibody rabbit anti-pERK (1:500; Santa Cruz, Carlsbad, CA, USA).

2.2.7 Statistics

All statistical analyses were performed using GraphPad Prism (version 5.0; San Diego, CA, USA). When comparing two groups, data were analyzed using unpaired Student's t test. Analysis of Variance (ANOVA) test was used for multiple comparisons followed by Bonferroni–corrected post hoc tests.

2.3 Results

2.3.1 Effect of CCK-8 on food intake.

<u>A- Effect of CCK-8 on thirty minute sucrose intake:</u>

Figure 2.4 shows daily 30 minutes sucrose intake at the beginning of the dark cycle. The rats started by drinking small amounts of sucrose as they were testing the new flavor offered to them. After few days, a baseline of intake was obtained. The effects of the three tested doses of CCK on 30 minutes sucrose intake are shown in Figure 2.4. Under our experimental conditions, a dose of 2 μg/kg did not cause a significant reduction in sucrose intake. A dose of 8 μg/kg resulted in approximately 30%

reduction of sucrose intake while rats receiving an intraperitoneal injection of 16 μg/kg of CCK-8 consumed about 60% of the sucrose amount consumed by the vehicle treated controls. Because of the robust nature of this later response, we elected to use this dose of CCK for further investigation.

B- Effect of CCK-8 (16 μg/kg, i.p.) on food intake:

As shown in Figure 2.5, intraperitoneal injection of CCK (16 μg/kg) caused a significant reduction of food intake by 30 minutes post injection. CCK treated groups ate 0.80 ±0.21 gm of chow compared to 2.46 ± 0.45 for vehicle treated controls. At 60 minutes after injection, CCK-8 failed to cause a significant reduction of food intake, though the mean intake was still 40% below that of vehicle control.

2.3.2 Effect of CCK and Ensure on plasma CCK concentration

At 30 min, CCK levels were 0.30 ± 0.11 ng/ml after CCK injection compared to 0.23 ± 0.02 ng/ml after Ensure® (n=4/group). Similarly, at 60 min CCK levels were 0.42 ± 0.10 ng/ml after CCK injection compared to 0.36 ± 0.02 ng/ml after Ensure® (n=4/group).

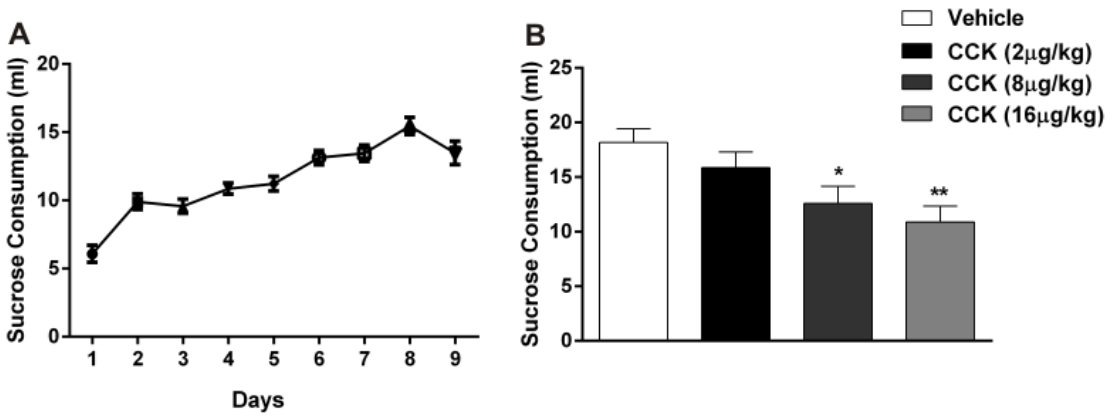


Figure 2.4: Effect of CCK-8 on sucrose intake

A: Baseline thirty minutes 15% sucrose intake (n=16).

B: Effect of 2, 8 and 16 microgram/kg of i.p. CCK-8 on 30 minutes sucrose intake. Data are presented as the mean ± S.E.M (n=4), *p<0.05 and ** p<0.01 represent a significant difference from the vehicle control.

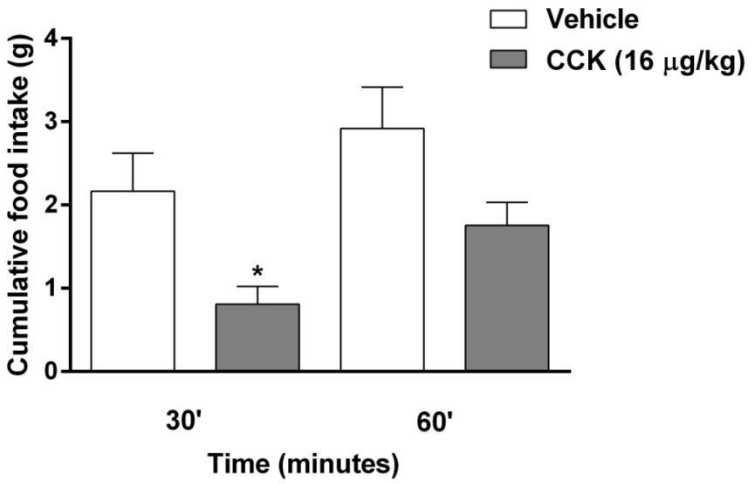


Figure 2.5: Effect of CCK-8 on food intake.

Bar graphs presenting the effect of i.p. CCK (16 μg/kg) on thirty and sixty min food intake. Data are presented as the mean ± S.E.M, *p<0.05 represents a significant difference from the respective control.

2.3.3 Effect of CCK-8 on c-Fos immunoreactivity.

Following experiments to determine the dose of CCK-8 that gives us an obvious food intake reducing effect, neuronal c-Fos expression as a marker of neuronal activity was used to study the effect of CCK-8 on the CVOs. The NTS and the AP were used as positive control regions for CCK induced neuronal activation. As shown in Figure 2.6, CCK induced robust c-Fos expression at the three levels of the NTS, the highest increase in c-Fos immunoreactivity was observed at the level of AP (bregma -3.68 to -14.8) followed by the rostral NTS (bregma -13.3 to -13.24). The caudal NTS (bregma -14.6 to -14.3) showed the lowest increase in c-Fos expression compared to vehicle treated animals. In addition to the NTS, CCK-8 induced a significant increase in c-Fos in the AP (Figure 2.7 A and C).

In the SFO (bregma -0.8 to -1.4), CCK-8 caused a significant increase in c-Fos immunoreactivity (18.6 nuclei ± 2.6) compared to the vehicle treated group (0.3 ± 0.3) as illustrated in Figure 2.7 C and D. According to the rostro-caudal organization, the SFO is classified into three regions; rostral, central and caudal. The central region is the richest in neuronal cell bodies and glial cells, however, rostral and caudal regions consist mainly of nerve fibers with a small number of cell bodies and glial cells. The core region of the SFO is more vascularized with abundant fenestrated capillaries, making this region potentially more accessible to circulating agents. Interestingly, we observed that the increase in c-Fos expression was more prominent in the core of the central region of the SFO (bregma -0.92 to -1.3, Figure 2.7 D).

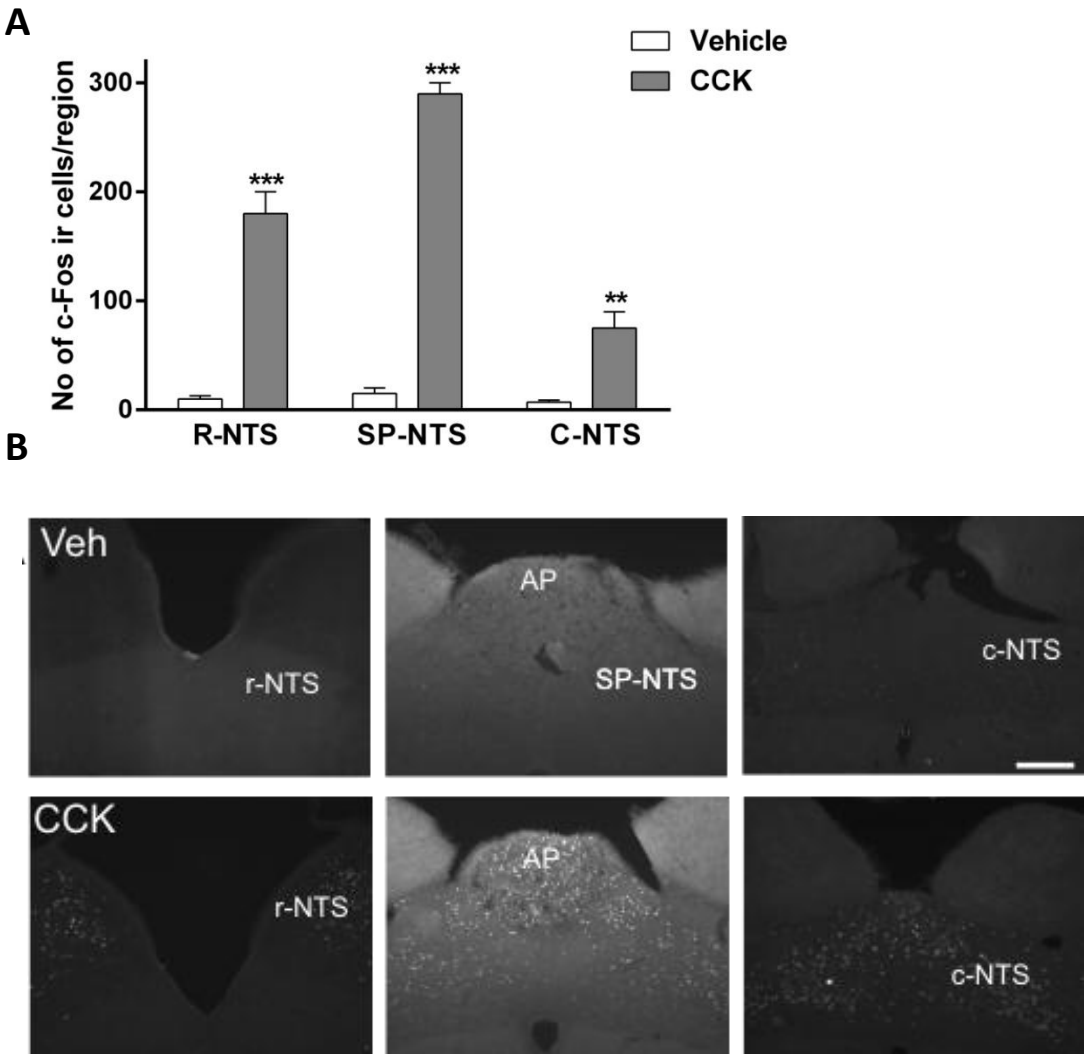


Figure 2.6: Effect of CCK-8 on c-Fos immunoreactivity in the NTS.

A: Effect of CCK-8 (16 μg/kg, i.p.) on c-Fos immunoreactivity at the coronal levels of the solitary tract nucleus (NTS). Data are presented as the mean ± S.E.M (n=4), *p<0.05, **p<0.01 and *** p<0.001 represent a significant difference from the vehicle control. B: Representative immunofluorescence micrographs showing c-Fos immunoreactivity in response to CCK (lower panel) at the three levels of the NTS. r-NTS: rostoral NTS, SP-NTS: subpostremal NTS and c-NTS: caudal NTS. Scale bar= 100 μm.

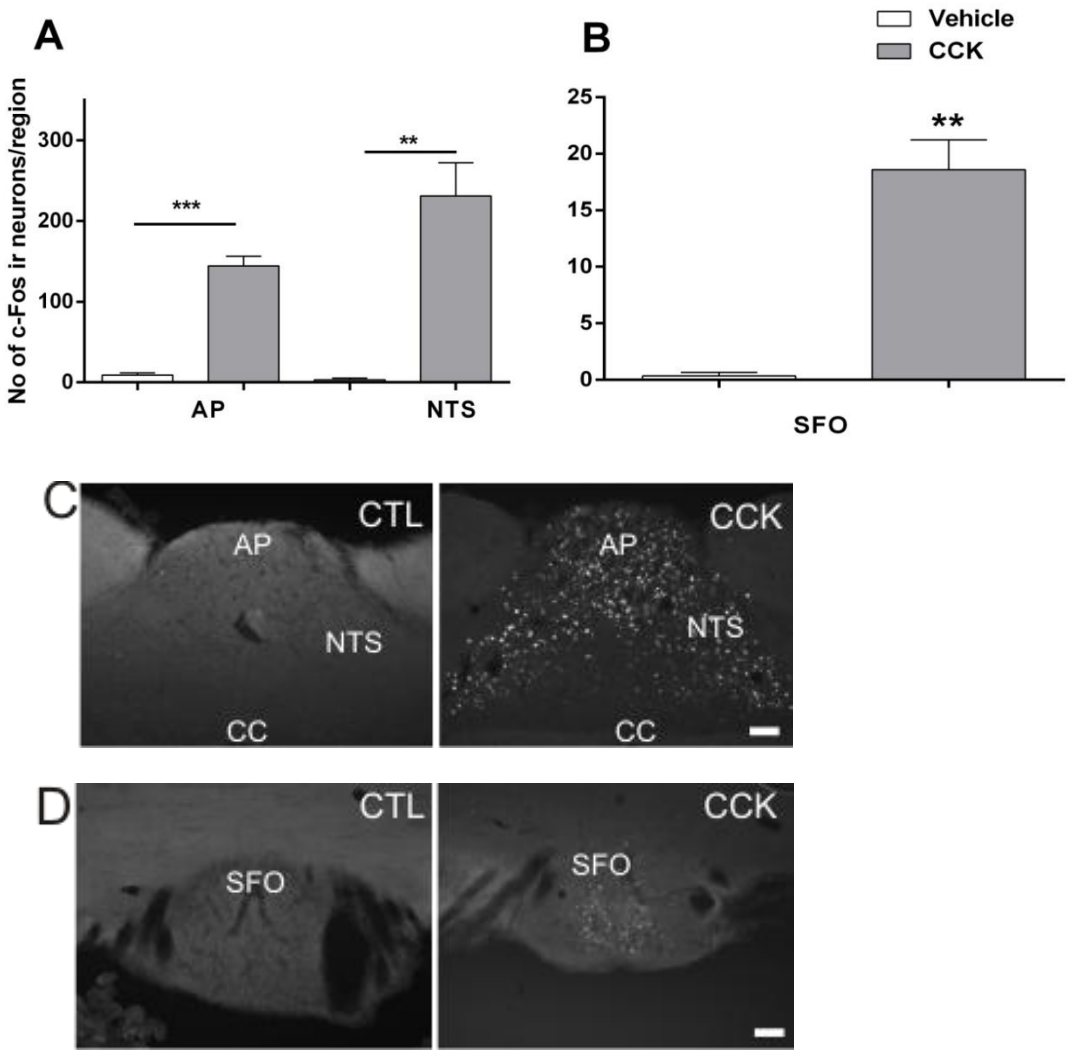


Figure 2.7: Effect of CCK-8 on c-Fos immunoreactivity in the brainstem and the SFO.

Bar graphs comparing the number of c-Fos immunoreactive cells in vehicle treated (white bars) versus CCK-treated animals in the AP/NTS (A) and in the SFO (B). Data are presented as the mean ± S.E.M (n=4), ** $p<0.01$ represents a significant difference from the vehicle control. *B:* Representative immunofluorescence micrographs showing changes in c-Fos immunoreactivity in response to CCK in the AP/NTS (C) and in the SFO (D). CTL: vehicle control, cc: central canal. Scale bar = 100 µm.

2.3.4 Effect of pretreatment with the $CCKR_1$ antagonist; devazepide on CCK-8 induced inhibition of food intake and c-Fos expression.

The next step was to examine the CCK receptor subtype responsible for CCK-8 induced c-Fos expression. This was achieved by testing the effects of pretreatment with the $CCKR_1$ antagonist; devazepide prior to CCK injection. First, I conducted a food intake study using a dose of devazepide based on previous reports [207-210]; 1 mg/kg was tested for attenuating the CCK food inhibitory action. As shown in Figure 2.8, devazepide at a dose of 1 mg/kg completely blocked the inhibitory effect of CCK on 30 minute food intake. In addition to this effect, and as was previously shown [211], devazepide alone resulted in a significant increase in food intake compared to vehicle treated control.

Then, the effect of pre-treatment with devazepide on CCK induced c-Fos in the brainstem and the SFO was investigated. In the brainstem regions, AP and NTS, pretreatment with devazepide significantly attenuated CCK-induced c-Fos expression (Figure 2.9 A and B). However, in the SFO, devazepide failed to reduce CCK-induced c-Fos and surprisingly treatment with devazepide alone resulted in a robust increase in c-Fos in the SFO (Figure 2.9 C and D). Thus, a second experiment was performed to test lower doses of devazepide for blocking the effect of CCK on food intake. Two doses of devazepide were used 300 and 600 μg/kg and only the latter dose significantly attenuated the inhibitory actions of CCK on food intake (Figure 2.10). Therefore a dose of 600 μg/kg was tested for its effect on CCK-induced c-Fos expression. As illustrated in Figure 2.11, devazepide (600 μg/kg) significantly reduced the CCK-induced increase in c-

Fos immunoreactive neurons in the AP (CCK; 68.5 ± 3.5 versus devazepide/CCK; 15.3 ± 5.7) and the NTS at the level of the AP (CCK; 128.0 ± 22.0 versus devazepide/CCK; 29.3 ± 11.5); however, it did not affect the number of CCK-induced c-Fos immunoreactive neurons in the SFO. Similar to the effect of 1mg/kg, devazepide at 600 μg/kg i.p. also increased c-Fos immunoreactivity in the SFO (Figure 2.11 C and D). However, the pattern of SFO c-Fos expression in devazepide treated animals was quite different from that of CCK treated animals. Devazepide treatment was associated with a diffuse, widespread c-Fos expression while c-Fos expression in response to CCK alone was confined to the central core of the SFO.

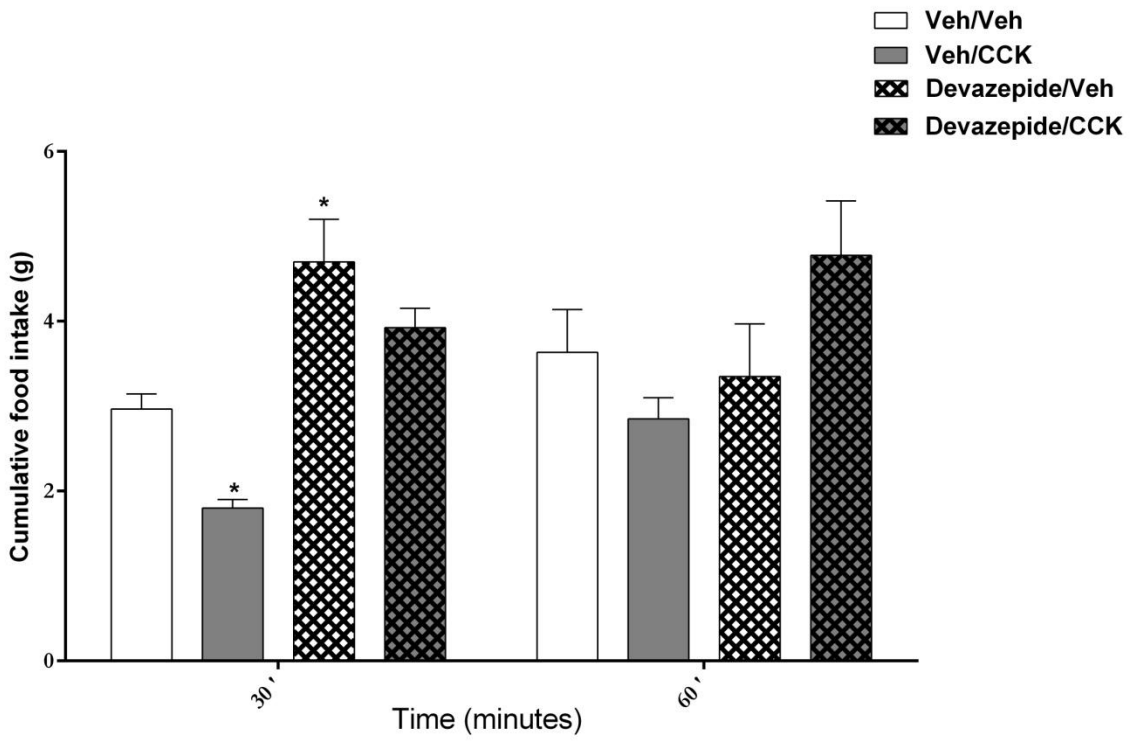
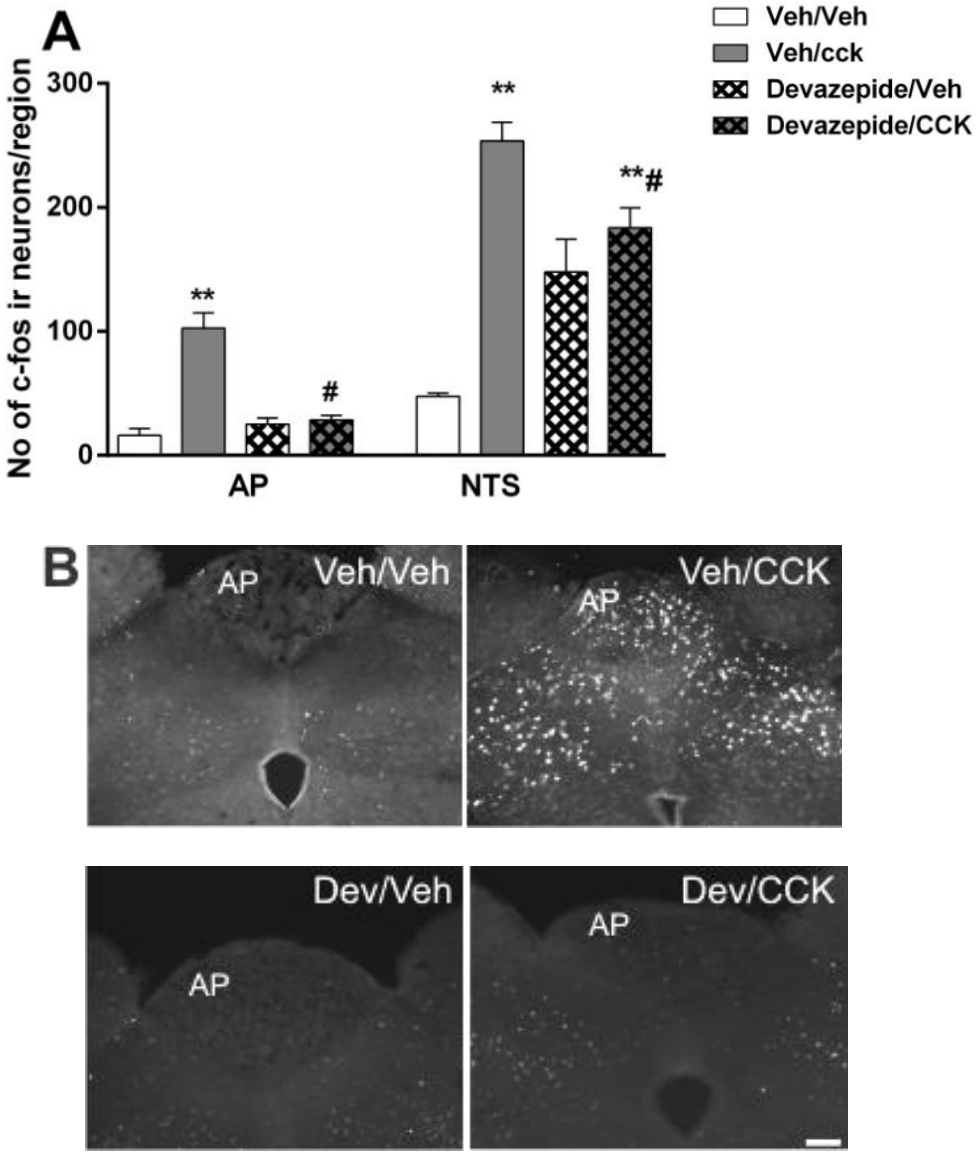


Figure 2.8: Effect of devazepide (1 mg/kg) on CCK induced inhibition of food intake.

Bar graphs presenting the effect of pretreatment with the $CCKR_1$ antagonist; devazepide in a dose of 1 mg/kg on CCK induced inhibition of food intake. Data are presented as the mean ± S.E.M (n=4), * $p<0.05$ represents a significant difference from the respective vehicle control.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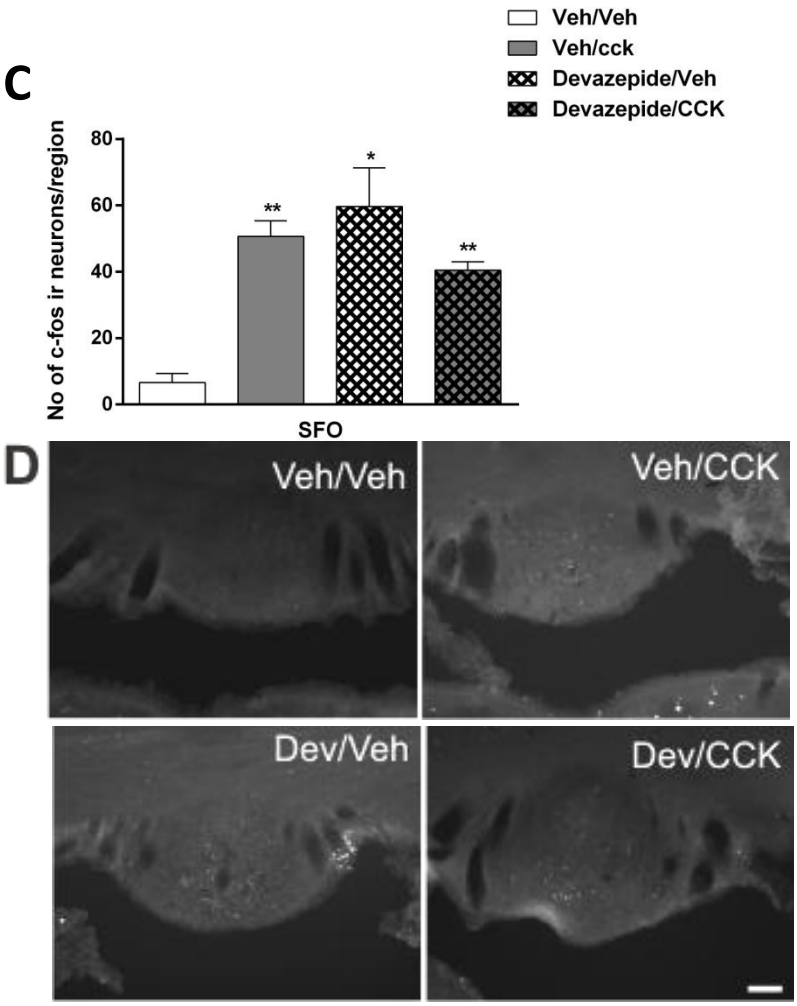


Figure 2.9: Effect of devazepide (1mg/kg) on CCK-induced c-Fos expression.

Number of c-Fos immunoreactive cells in four different groups treated with vehicle (Veh/Veh), CCK (16 µg/kg; Veh/CCK), devazepide (1 mg/kg; Devazepide/Veh) and a combination of both (Devazepide/CCK) in the AP/ NTS (**A**) and in the SFO (**C**). Data are presented as the mean ± S.E.M (n=4), *p<0.05, ** p<0.01 and *** p<0.001 represent a significant difference from the respective vehicle control. # p<0.05 represents a significant difference from the respective CCK treated group. Representative immunofluorescence micrographs for the four treatment groups in the AP/ NTS and the SFO are shown in **B** and **D**, respectively. Scale bar = 100 µm.

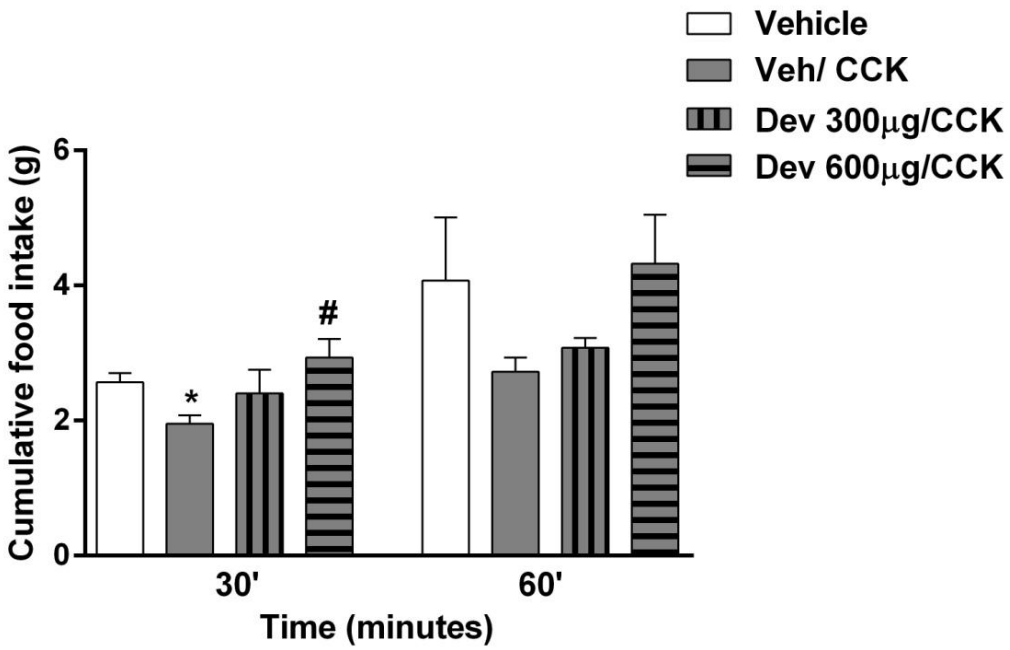
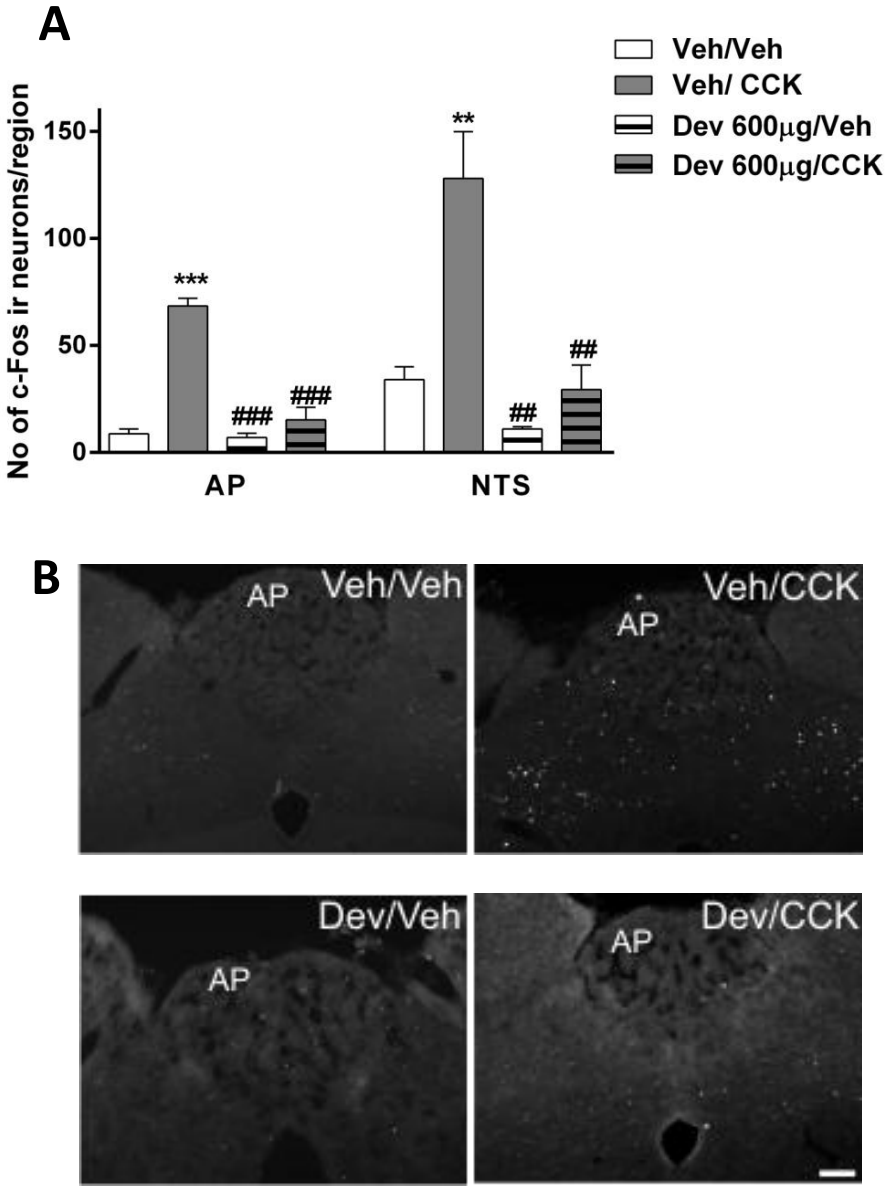


Figure 2.10: Effect of lower doses of devazepide on CCK-induced inhibition of food intake.

Bar graphs presenting the effect of pretreatment with devazepide (300 and 600 μg/kg) on CCK induced inhibition of food intake. Data are presented as the mean ± S.E.M (n=4), * $p<0.05$ represents a significant difference from the respective vehicle control. # $p<0.05$ represents a significant difference from the respective CCK treated group.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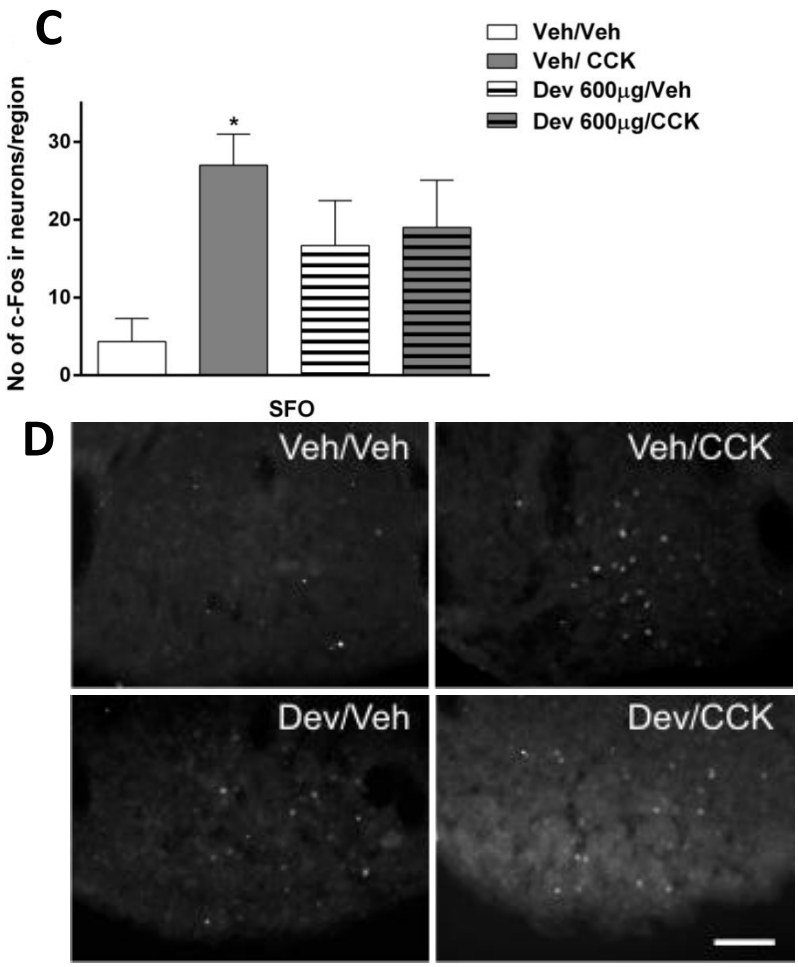


Figure 2.11: Effect of devazepide (600 μg/kg) on CCK-induced c-Fos expression.

Number of c-Fos immunoreactive cells in four different groups treated with vehicle (Veh/Veh), CCK (16 μg/kg; Veh/CCK), devazepide (600 μg/kg; Dev 600 μg /Veh) and a combination of both (Dev 600 μg /CCK) in the AP/ NTS (**A**) and in the SFO (**B**). Data are presented as the mean ± S.E.M (n=4), * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ represent a significant difference from the respective vehicle control. ## $p<0.01$ and ## $p<0.001$ represent a significant difference from the respective CCK treated group. Representative immunofluorescence micrographs for the four treatment groups in the AP/ NTS and the SFO are shown in **C** and **D**, respectively. Scale bar = 100 μm.

2.3.5 Effect of pretreatment with the $CCKR_2$ antagonist; L-365.260 on CCK-8 induced inhibition of food intake and c-Fos expression.

Showing that CCK-induced c-Fos in the SFO is not mediated by $CCKR_1$, the effect of $CCKR_2$ antagonist, L-365.260 was tested. Similar to what was done previously, a dose of L-365.260 was chosen according to previous reports [212] and was tested for its effect in CCK-mediated reduction in food intake. As shown in Figure 2.12, L-365.260 in a dose of 500μg/kg was unable to block CCK-induced reduction of feeding, as expected. When the c-Fos study was performed, a robust c-Fos expression was noticed in the brainstem regions; AP and NTS as well as in the SFO (Figure 2.13). Similar to the procedure followed with devazepide, two lower doses (100 and 300 μg/kg) of L-365.260 were tested for their effect on CCK-induced reduction of food intake, as well as c-Fos expression. As shown in Figure 2.14, both doses of L-365.260 failed to show any effect on the inhibitory action of CCK on food intake. Consistent with previous work, L-365.260 alone did not cause an increase in food intake [213]. Accordingly, the lower dose was used to test its effect on c-Fos immunoreactivity. As shown in Figure 2.15 (A and B), L-365.260 (100μg/kg) significantly reduced the CCK-induced increase in the number of c-Fos immunoreactive neurons in the AP (CCK; 52.5 ± 2.5, versus L-365,260/CCK; 15.5 ± 2.5) and the NTS (CCK; 66.5 ± 12.5, versus L-365,260/CCK; 116.0 ± 9.0). In the SFO (Figure 2.15 C and D), L-365.260 also attenuated CCK-induced c-Fos immunoreactivity (L-365.260/CCK; 2.5 ± 1.5, versus CCK; 18.0 ± 3.0).

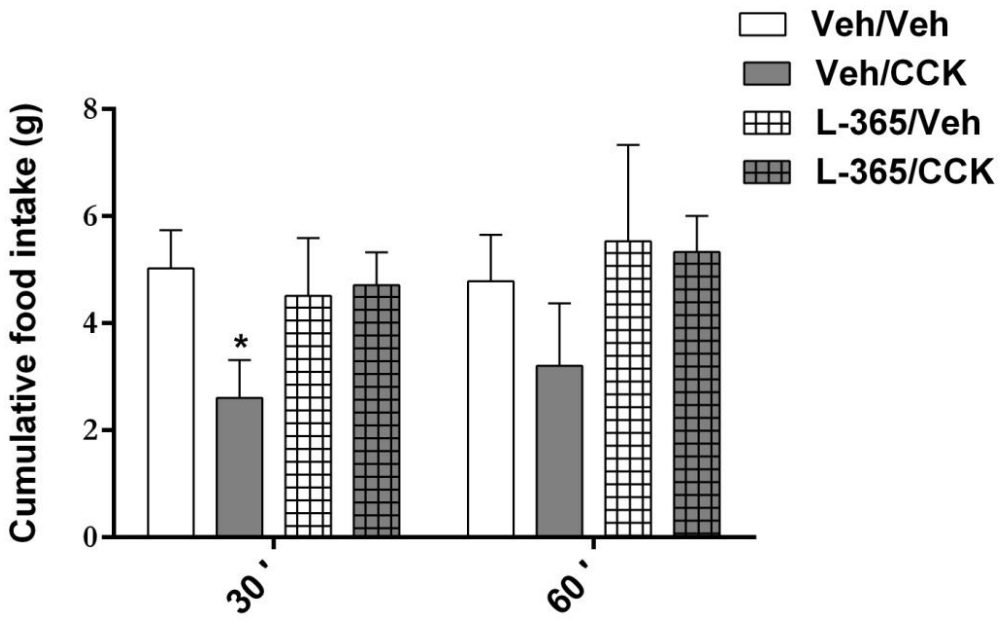
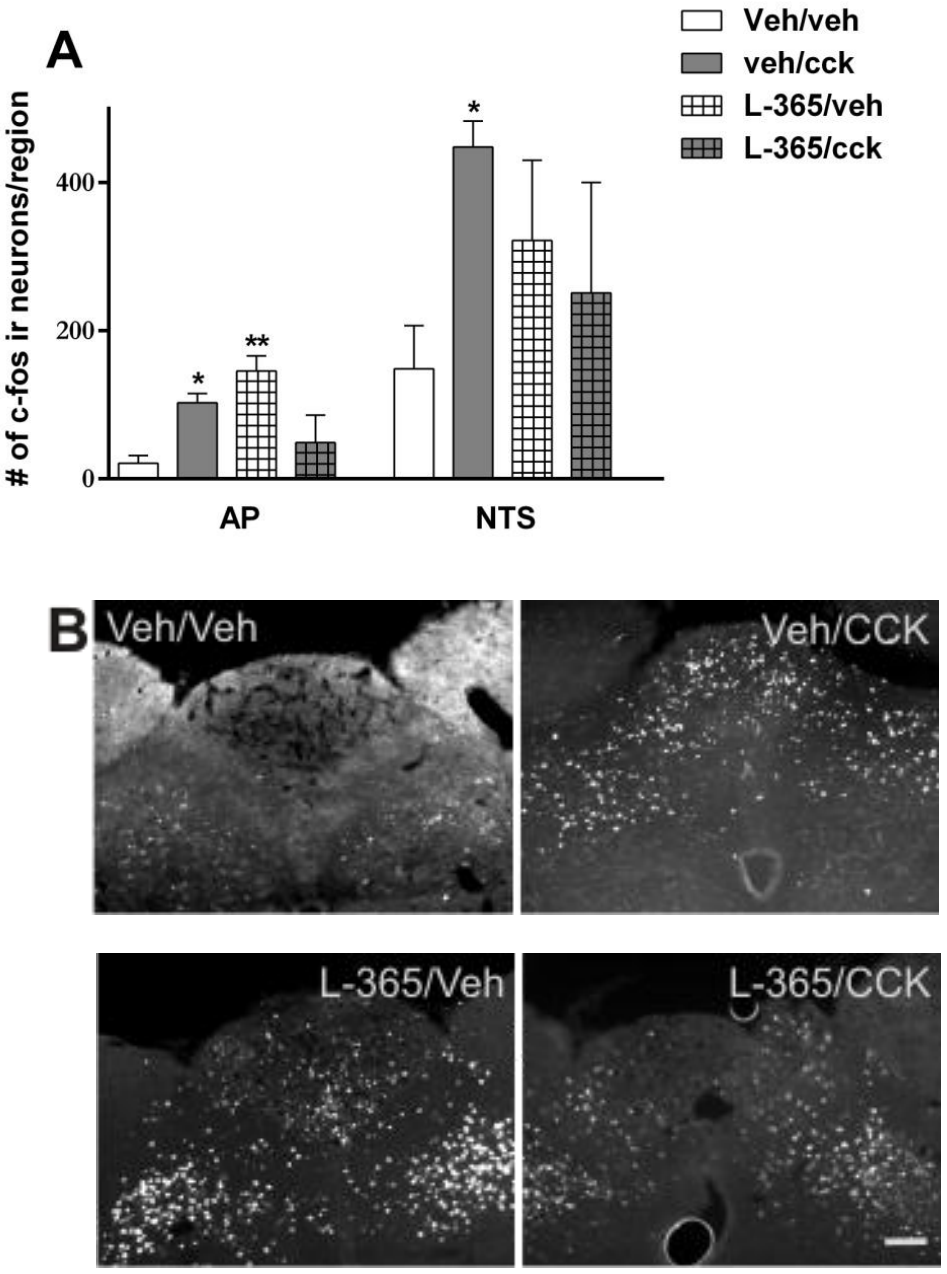


Figure 2.12: Effect of L-365.260 (0.5 mg/kg) on CCK induced inhibition of food intake.

Bar graphs presenting the effect of pretreatment with the $CCKR_2$ antagonist; L-365.260 in a dose of 0.5 mg/kg on CCK induced inhibition of food intake. Data are presented as the mean ± S.E.M (n=4), * $p<0.05$ represents a significant difference from the respective vehicle control.


A
Veh/veh
veh/cck
L-365/veh
L-365/cck
# of c-fos ir neurons/region
400
200
0
*
**
*
AP
NTS
B
Veh/Veh
Veh/CCK
L-365/Veh
L-365/CCK

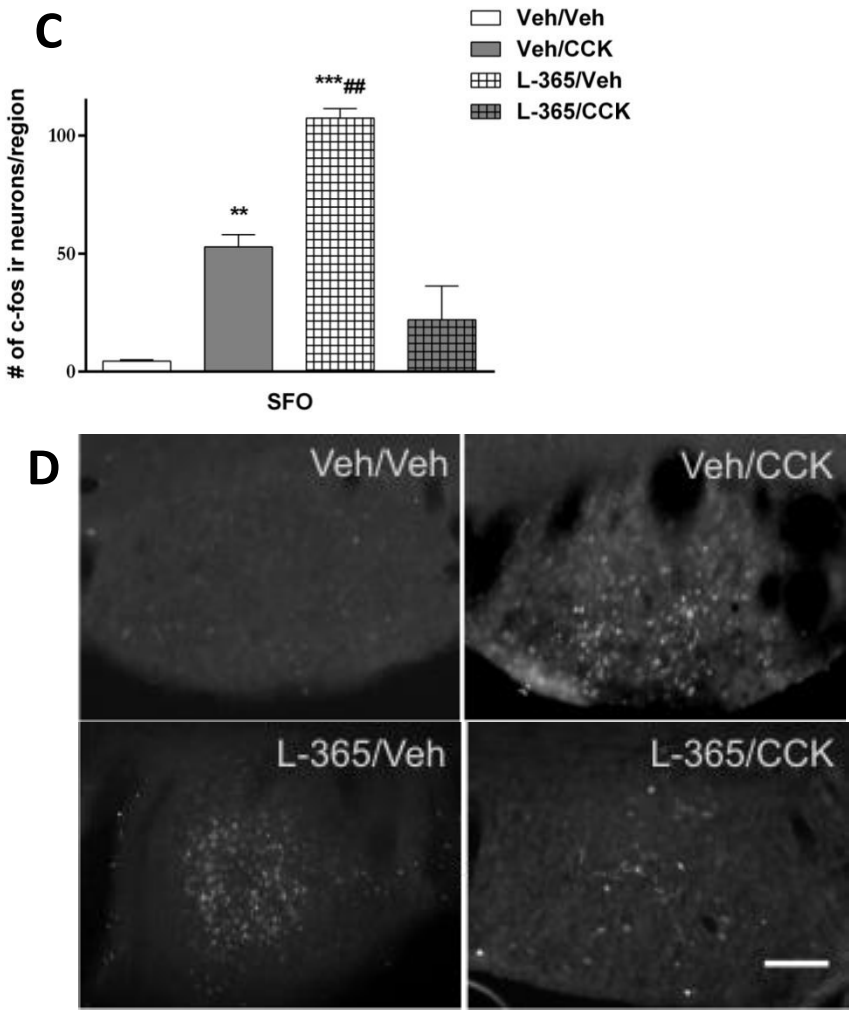


Figure 2.13: Effect of L-365.260 (0.5mg/kg) on CCK-induced c-Fos expression.

Number of c-Fos immunoreactive cells in four different groups treated with vehicle (Veh/Veh), CCK (16 µg/kg; Veh/CCK), L-365.260 (0.5 mg/kg; L-365/Veh) and a combination of both (L-365/CCK) in the AP/ NTS (**A**) and in the SFO (**C**). Data are presented as the mean ± S.E.M (n=4), * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ represent a significant difference from the respective vehicle control. ## $p<0.01$ represents a significant difference from the respective CCK treated group. Representative immunofluorescence micrographs for the four treatment groups in the AP/ NTS and the SFO are shown in **B** and **D**, respectively. Scale bar = 100 µm.

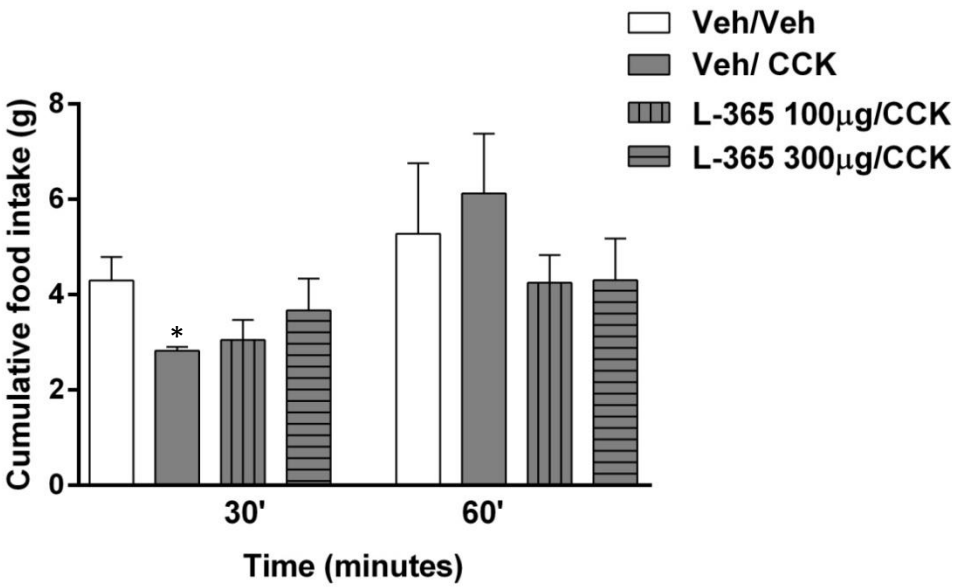
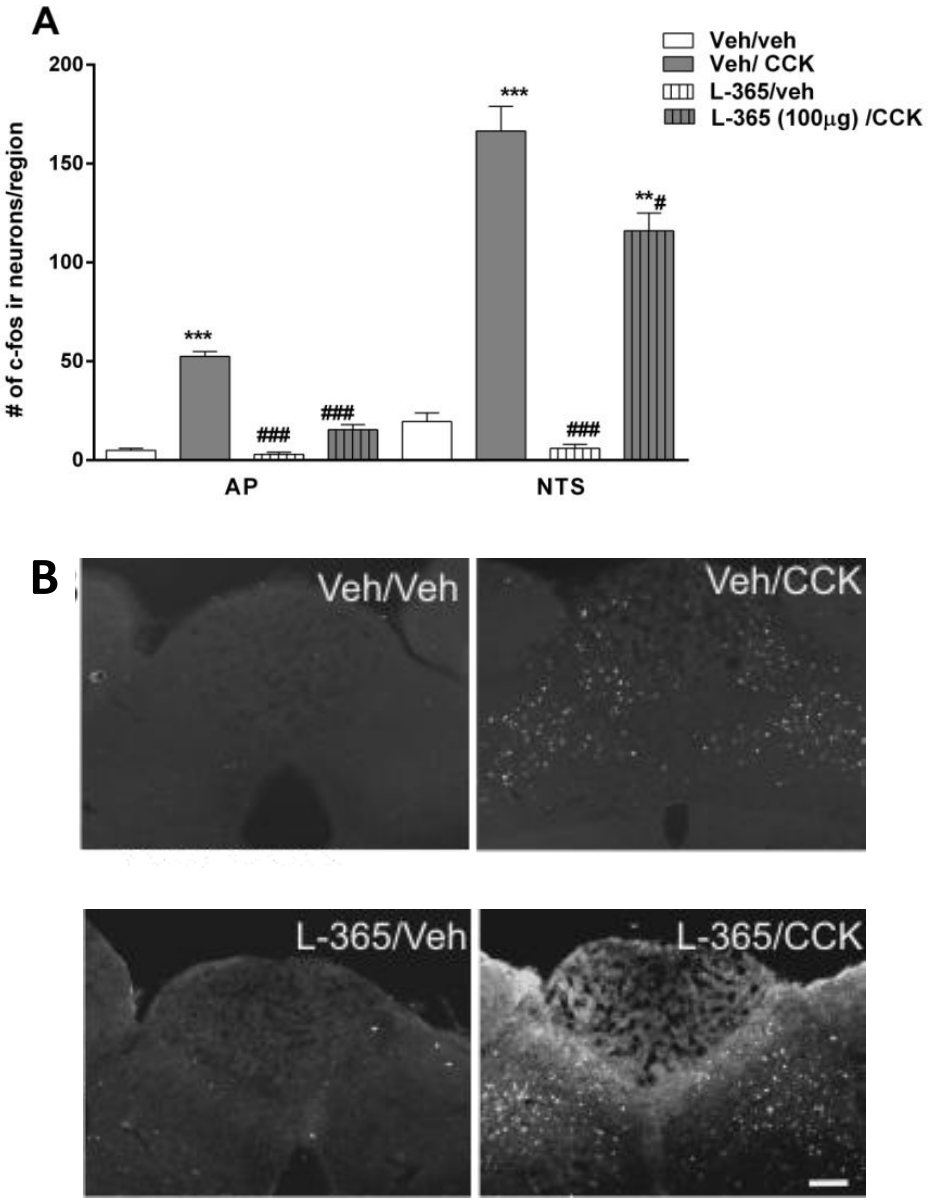


Figure 2.14: Effect of lower doses of L-365.260 on CCK-induced inhibition of food intake.

Bar graphs presenting the effect of pretreatment with L-365.260 (100 and 300 μg/kg) on CCK induced inhibition of food intake. Data are presented as the mean ± S.E.M (n=4).


A
Veh/veh
Veh/ CCK
L-365/veh
L-365 (100μg) /CCK
# of c-fos ir neurons/region
200
150
100
50
0
***
***
**#
###
###
###
AP
NTS
B
Veh/Veh
Veh/CCK
L-365/Veh
L-365/CCK

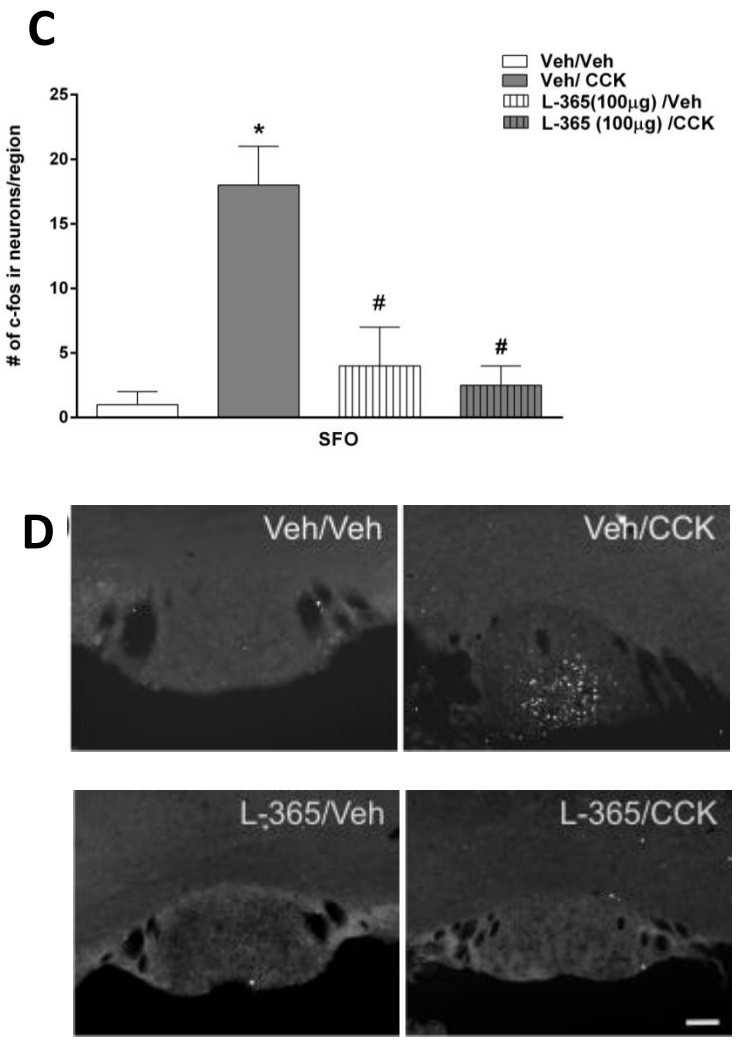
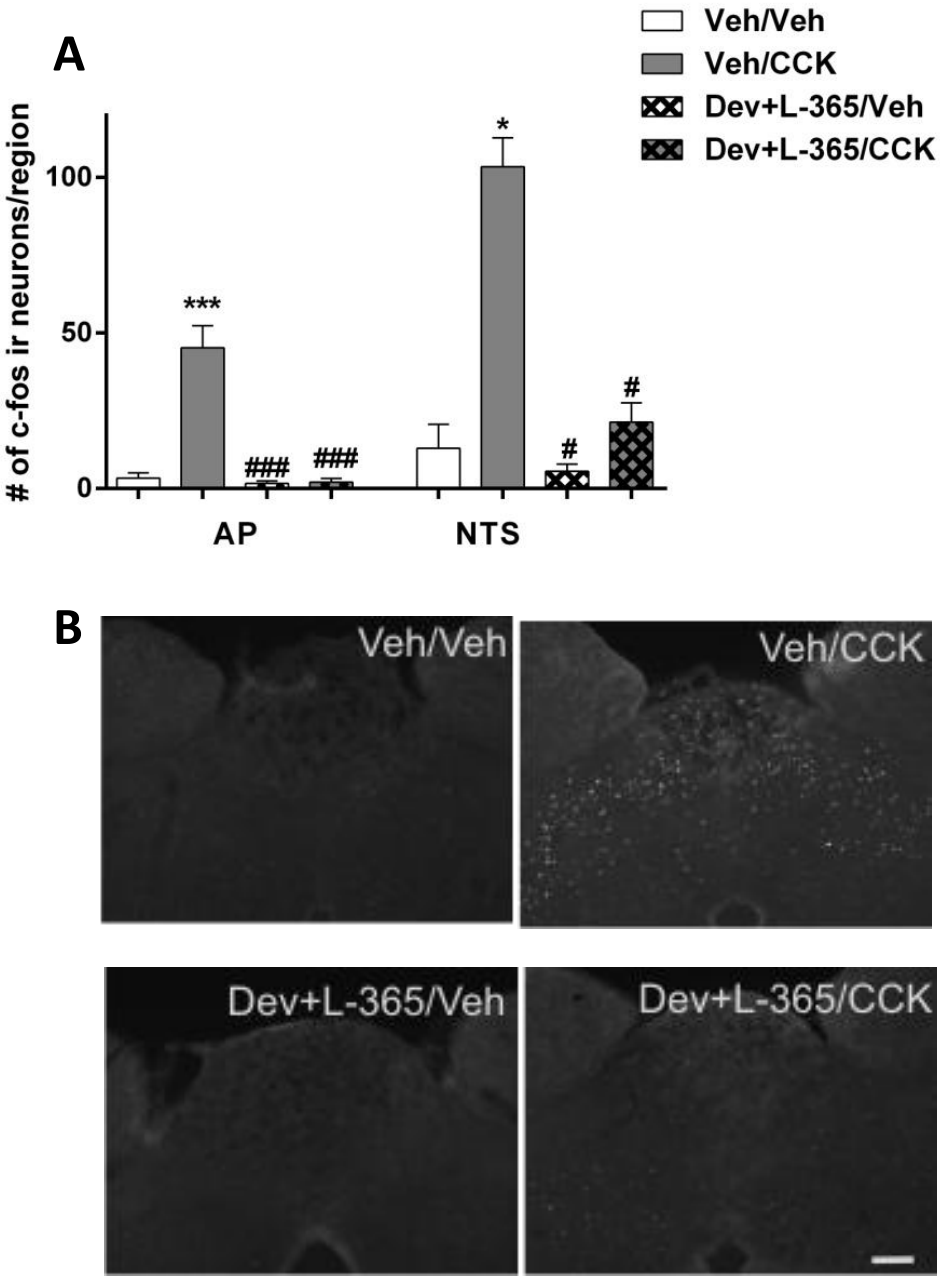


Figure 2.15: Effect of L-365.260 (100 μg/kg) on CCK-induced c-Fos expression.

Bar graphs comparing the number of c-Fos immunoreactive cells in four different groups treated with vehicle (Veh/Veh), CCK (16 μg/kg; Veh/CCK), L-365.260 (300 μg/kg; L-365 100 μg /Veh) and a combination of both (L-365 100 μg /CCK) in the AP/ NTS (A) and in the SFO (B). Data are presented as the mean ± S.E.M (n=4), * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ represent a significant difference from the respective vehicle control. # $p<0.01$ and ### $p<0.001$ represent a significant difference from the respective CCK treated group. Representative immunofluorescence micrographs for the four treatment groups in the AP/ NTS and the SFO are shown in C and D, respectively. Scale bar = 100 μm.

2.3.6 Effect of pretreatment with a combination of devazepide and L-365.260 on CCK-8-induced c-Fos expression.

When both $CCKR_1$ and $CCKR_2$ receptor antagonists were administered prior to CCK-8 treatment, c-Fos expression in the AP and the NTS was attenuated to the levels of the vehicle treated group (Figure 2.16 A and C). Interestingly, in the SFO, combining the two antagonists failed to block the CCK induced c-Fos expression (Figure 2.16 B and D). When the two antagonists were given without CCK, they resulted in a significant increase in c-Fos immunoreactivity in the SFO (veh/veh; 2.3 ± 0.6, versus devazepide+L-365.260; 11.25 ± 2.2, Figure 2.16 B and D).


A
Veh/Veh
Veh/CCK
Dev+L-365/Veh
Dev+L-365/CCK
# of c-fos ir neurons/region
100
50
0
***
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AP
NTS
B
Veh/Veh
Veh/CCK
Dev+L-365/Veh
Dev+L-365/CCK

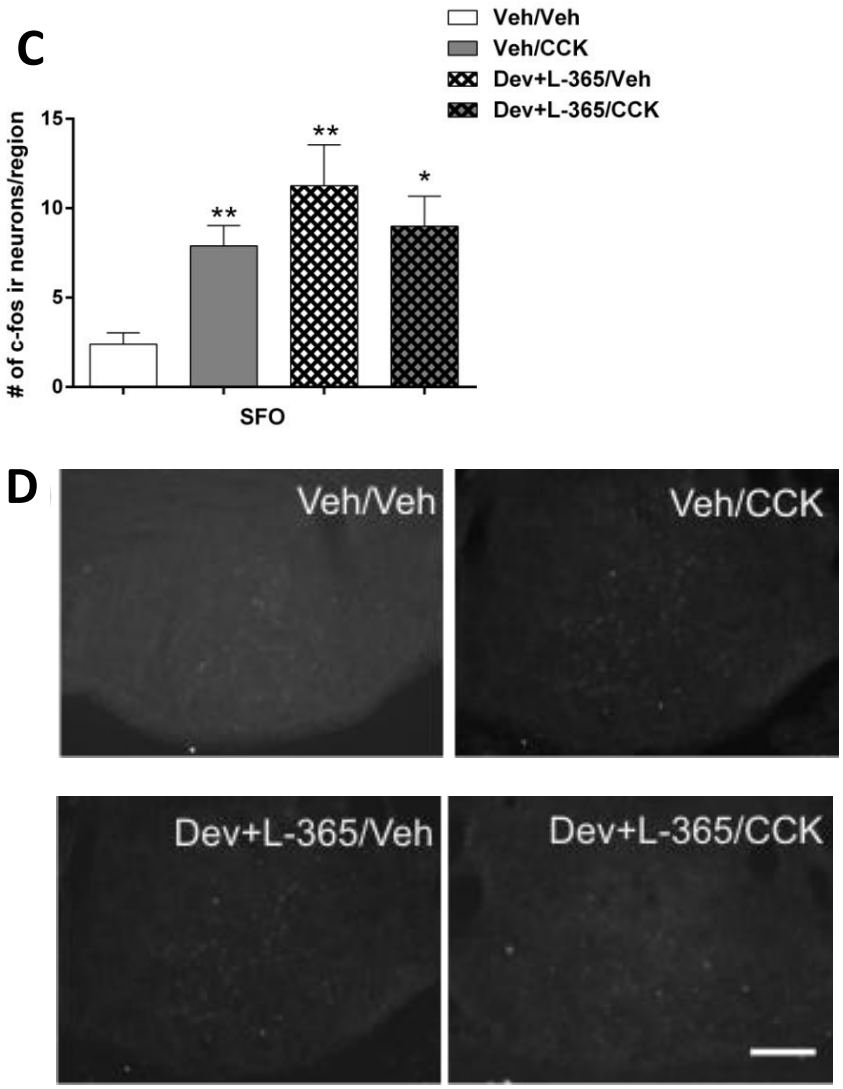


Figure 2.16: Effect of combining devazepide (600 μg/kg) and L-365.260 (100 μg/kg) on CCK-induced c-Fos expression.

Bar graphs comparing the number of c-Fos immunoreactive cells in four different groups treated with vehicle (Veh/Veh), CCK (16 μg/kg; Veh/CCK), devazepide+ L-365.260 (600 and 100 μg/kg; Dev+L-365/Veh) and a combination of both (Dev+L-365/CCK) in the AP/ NTS (A) and in the SFO (C). Representative immunofluorescence micrographs for the four treatment groups in the AP/ NTS and the SFO are shown in B and D, respectively. Scale bar = 100 μm.

2.3.7 Effect of CCK-8 on p-ERK immunoreactivity.

Previous reports have shown that the inhibitory effect of CCK on food intake is mediated through the activation of the ERK1/2 pathway in the brainstem (mainly the NTS) [168, 201], therefore, we investigated whether activation of SFO neurons by CCK is mediated through the same pathway. As shown in Figure 2.17 A and represented in Figure 2.17 B, treatment with CCK-8 significantly increased the number of p-ERK immunoreactive neurons in the NTS as well as in the SFO. In the NTS, the number of p-ERK immunoreactive cells in the CCK-treated group was 27.0 ± 4.00 compared to 8.0 ± 2.6 in vehicle treated rats. The number of p-ERK immunoreactive cells in the SFO in response to CCK was 18.5 ± 4.0 compared to 4.3 ± 0.6 in response to vehicle. The pattern of p-ERK activation which was distributed peripherally in the SFO (mainly in neurons, as well as some nerve fibers), was different from that observed with c-Fos which was localized to the central core of the SFO.

2.3.8 Effect of $CCKR_1$ and $CCKR_2$ antagonists on CCK-induced p-ERK immunoreactivity.

To study the CCK receptor subtype mediating the observed increase in p-ERK immunoreactivity in the NTS and in the SFO, we compared p-ERK immunoreactivity in response to CCK-8 alone or in combination with devazepide or L-365,260. In the NTS, CCK-induced increase in p-ERK immunoreactivity was attenuated by combination of CCK with devazepide or L-365,260. In the SFO, devazepide did not alter the effects of CCK on p-ERK immunoreactivity while L-365,260 reduced the responses to CCK. Consistent with the c-Fos data, devazepide alone induced a significant increase in p-ERK immunoreactivity in the SFO (Figure 2.18).

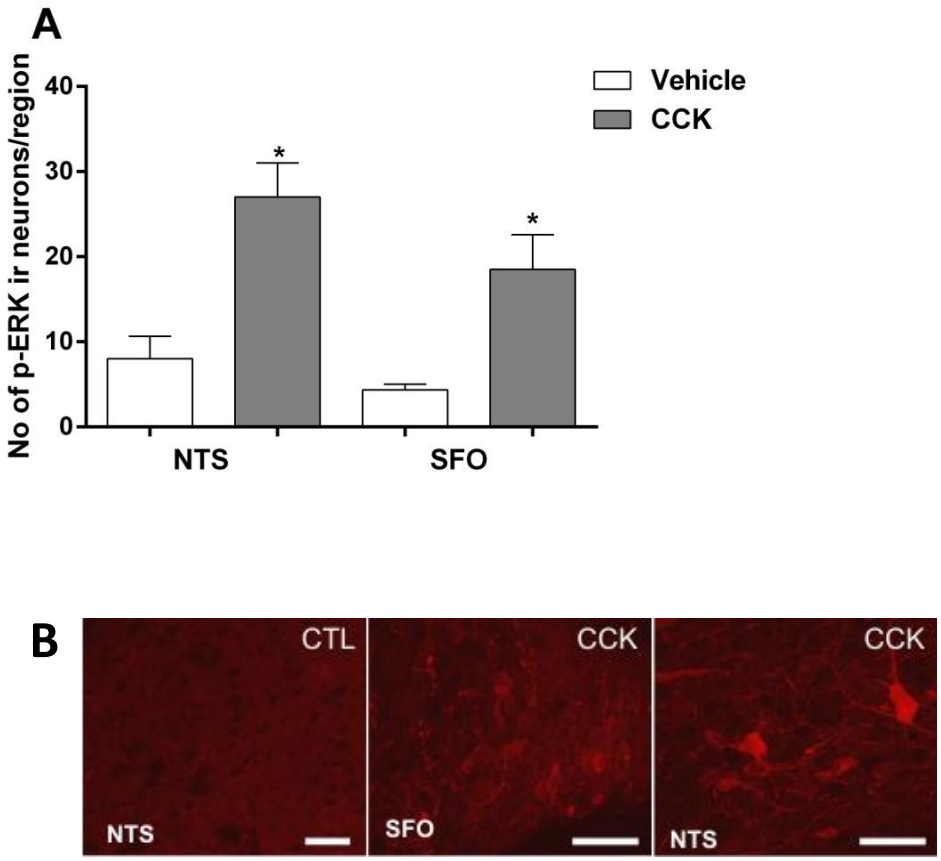


Figure 2.17: Effect of CCK-8 on p-ERK immunoreactivity in the NTS and the SFO.

A: Bar graphs comparing the number of p-ERK immunoreactive cells in response to CCK-8 or vehicle in the NTS and in the SFO.

B: Representative confocal microscopic images showing no p-ERK expression in the NTS in vehicle treated group (CTL) and p-ERK immunoreactivity in the SFO and in the NTS in response to CCK treatment, respectively. Scale bar = 50 µm.

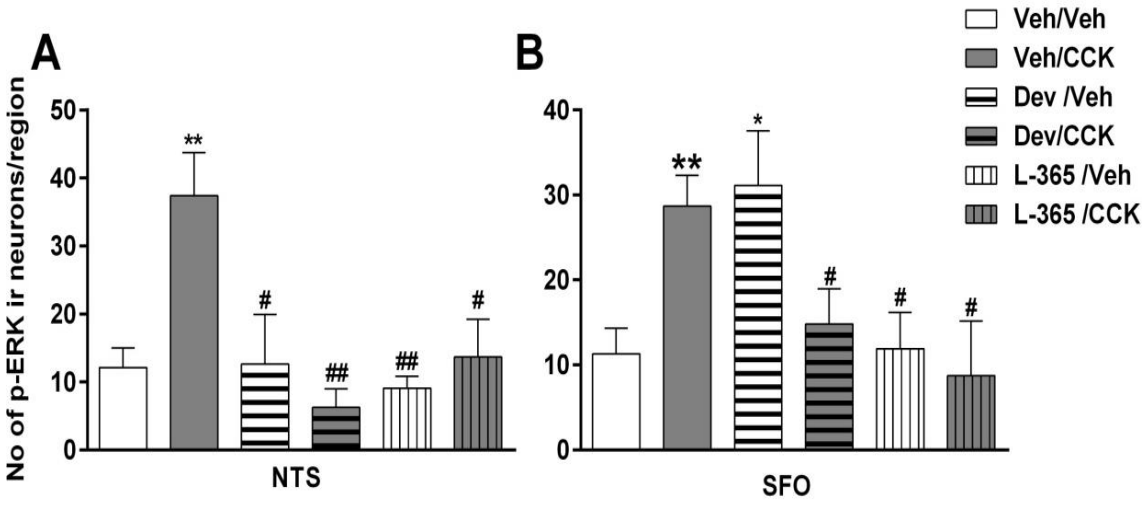


Figure 2.18: Effect of CCKR antagonists on CCK-induced p-ERK.

Bar graphs comparing the number of p-ERK immunoreactive cells in six different groups treated with vehicle (Veh/Veh), CCK (16 µg/kg; Veh/CCK), devazepide (600 µg/kg; Dev/Veh), devazepide + CCK (Dev/CCK), L-365,260 (100 µg/kg; L-365/Veh) and L-365,260 + CCK (L-365/CCK) in the NTS (A) and in the SFO (B).

Data are presented as the mean ± S.E.M (n = 4), *$p<0.05$ and ** $p<0.01$, represent a significant difference from the respective control. $^{\#}p<0.05$ and $^{\#\#}$ $p<0.01$ represent a significant difference from respective CCK treated group.

2.4 Discussion

In the present Chapter I have provided evidence that the sensory CVOs namely, the AP and the SFO are sites of action of circulating CCK. The AP, used as a positive control region, is a previously studied site of action of CCK [109, 131, 136, 214]. In this Chapter, I was able to replicate previous work using c-Fos as a marker of neuronal activation at this region [128, 215-217] as well as the involvement of both $CCKR_1$ and $CCKR_2$ in CCK mediated effect on the AP [109, 218, 219]. The new finding of this Chapter is that the SFO represents another potential sensory CVO site at which circulating CCK may act to exert its physiological actions. The activation of c-Fos expression in the SFO in response to a systemic administration of CCK demonstrates the ability of SFO neurons to respond to circulating CCK-8, which does not readily cross the blood brain barrier [167]. Moreover, the increase in p-ERK immunoreactivity in response to CCK represents further evidence that CCK activates neurons in the SFO, using a similar signaling pathway to that reported in the NTS [201]. The effect of CCK on the SFO is mediated by a $CCKR_2$ pathway demonstrated by the ability of a selective $CCKR_2$ antagonist; L-365.260 to attenuate CCK-induced c-Fos and p-ERK immunoreactivity. These data suggest that the SFO may act as a novel site of action for circulating CCK.

There are numerous studies using both receptor autoradiography and in situ hybridization that have examined the distribution of CCK receptors in the brain and have not reported the presence of CCK receptors in the SFO [218-222]. This is probably due to their low level of expression as a gene chip analysis revealed low levels of CCK receptor expression in the SFO [144] showing the presence of CCK receptors in the SFO

in a lower density compared to other neurotransmitter receptors in the SFO. In this study, it is very obvious that CCK receptor gene expression is present at lower expression levels compared to other receptors, e.g. endothelin B and 5-HT_2C [144]. The SFO being a very small area is easily lost in sectioning and may have been overlooked in CCK receptors localization studies. However, looking at the Allen Mouse Brain Atlas for CCK receptors, both subtypes are clearly present in the SFO as shown by in situ hybridization in the coronal sections of this reference guide. Moreover, recent work has shown the presence of both CCK_1 and CCK_2 receptor mRNA in the SFO, while electrophysiological studies indicate that about 50% of SFO neurons respond to CCK, with the majority showing rapid reversible depolarization [223].

c-Fos immunoreactivity was used as a marker of neuronal activity. c-Fos is an immediate early gene that is expressed by activated neurons [197]. Although c-Fos measurement is a well-established semi-quantitative method of measuring neuronal activation, its use has some important limitations, thus when using c-Fos as a marker, one should consider a number of points. Firstly, there is a critical time in which c-Fos is maximally expressed in response to a stimulus. In these experiments, animals were studied 90 minutes after CCK administration, since maximal c-Fos expression in response to a stimulus [224] or specifically CCK has been shown to be between 90-120 minutes [198-200]. Secondly, exposure to stress induces c-Fos expression [225, 226], so it is important to adapt the animals to handling and manipulation to decrease the levels of basal c-Fos expression. The low level of c-Fos expression in all vehicle treated animals is indicative that our animals underwent careful conditioning and were not stressed at

the time of the study. One important limitation of using c-Fos is interpreting negative results. Absence of c-Fos might indicate the certain population of neurons express other immediate early genes or they may need higher thresholds of stimulation to express c-Fos [227]. Another major limitation of this technique is that c-Fos does not mark cells with inhibitory synaptic or transcriptional drive [228]. Therefore, it is very important to keep these limitations in mind while interpreting c-Fos results.

The dose of CCK-8 used (16 μg/kg) was selected as one that induced a significant reduction of food intake following intraperitoneal administration. It was also chosen as it gave circulating levels in plasma of a similar magnitude to that of an Ensure test meal. The ELISA kit used measures total plasma c-terminal octapeptide which explains the higher values we obtained (nMol) compared to the other cited literature showing plasma CCK concentrations at the pMol level [229]. I did not perform any separation of CCK-8 (from gastrin) to be able to get concentrations in the picomolar ranges. Nonetheless, I am aware of the limitations of the assay I used and the results shown here are not to determine the circulating levels of CCK. Determination of plasma CCK was intended for a purely comparative purpose to see if the used CCK dose will give higher values of circulating peptide compared to a liquid Ensure meal. In fact, the results presented here match well with other studies that used the same ELISA kit [202, 205, 230].

Although the dose of CCK we used may not mimic physiological patterns of release following a meal, we believe that our findings are consistent with previous work examining regional brain activation by circulating peptides. Previous studies have used

lower doses of CCK to reduce food intake (<4 μg/kg) [42, 231], however, under our experimental conditions we did not observe a significant reduction in feeding using 2 μg/kg , as has also been reported by others [232]. In that study, the minimal effective dose for CCK-8 to produce anorexia was 25 nmol/kg (equivalent to 29 μg/kg) when they used CCK-8s from one supplier and 50 nmol/kg was the minimum effective dose from another supplier. So, different sources of the sulphated form of the peptide might have an impact on the biological activity of the preparation used for injection, hence, the physiological response. In fact, there is one publication that used CCK from the source I used in my feeding studies and the authors used a CCK dose of 50 μg/kg [233].

It is important to note that several forms of CCK have been detected in the plasma for example, CCK-8, CCK-22, and CCK-58 [234-236]. However, using techniques that minimize CCK peptide degradation, CCK-58 is the only circulating form of CCK in the rat [229, 237]. CCK-58 produces a prolonged inhibition of food intake compared to CCK-8, inhibiting food intake for as long as 60 min, and a longer latency to first meal and greater intermeal interval [238, 239]. However, CCK-58 is not commercially available which explains the more common use of the octapeptide form in research. Further work on the actions of the physiological forms of CCK on the SFO is still required.

An important consideration with an action on reduced food intake is whether the effects observed are due to malaise. This has previously been examined in regard to CCK. Ervin *et al*., [240], showed that the aversive effect of CCK is related to the route of administration and is different in rats compared to humans. The lowest dose they used was 25 nmol/kg i.p. (about 28.6 μg/kg), which did not produce taste aversion when

administered intraperitoneally, suggesting the animals were not suffering malaise. In addition, McCutcheon *et al*. [241], measured aversion by studying pica behavior (ability to cause kaolin eating) as a measure of gastric distress. They concluded that a dose of 20 μg/kg of CCK did not produce pica. Since the dose of CCK used is below these levels, it is unlikely that the activation of neurons in the SFO by CCK is due to malaise.

The AP and the NTS were used as positive control regions for CCK-8 induced c-Fos activation in response to i.p. injection of CCK-8. The distribution of c-Fos immunoreactivity in the brainstem was entirely consistent with previous reports [242-245]. The c-Fos-staining was stronger in the rostral part of the nucleus of the solitary tract than its caudal counterpart with the sub-postremal NTS showing the highest activation levels [242-245].

c-Fos expression in the SFO was confined to the central core of the SFO, a region which anatomical and tracing studies have demonstrated to be characterized by an abundance of fenestrated capillaries as well as cell bodies of output neurons [246]. This pattern of distribution of c-Fos immunoreactive neurons is highly suggestive of a direct effect of circulating CCK on SFO neurons with likely projections to hypothalamic autonomic control nuclei.

The next step was to identify the CCK receptor subtype mediating this response, by examining the effect of devazepide (CCK_1 receptor antagonist), and L-365.260 (CCK_2 receptor antagonist), on CCK induced c-Fos expression in the SFO, as well as in the brainstem. As previously reported [242, 247], devazepide blocked CCK induced c-Fos in the AP and NTS, however, it did not affect SFO c-Fos expression. These findings suggest

that CCK induced c-Fos in the SFO is not mediated by the CCK_1 receptor. On the other hand, blockade of CCK_2 receptor by L-365.260 resulted in attenuation of the number of c-Fos immunoreactive neurons in the SFO, as well as in the brainstem, supporting a role for the CCK_2 receptor in mediating depolarization in these regions. Interestingly, rats treated with devazepide alone had increased c-Fos immunoreactivity in the SFO but not in the brainstem. A possible explanation of this effect is that CCK_1 receptor stimulation might be activating neurons that send inhibitory signals to other neuronal populations, thus blocking CCK_1 using devazepide is releasing this inhibitory input leading to activation of these neurons, subsequently leading to the expression of c-Fos. The pattern of devazepide induced c-Fos expression, combined with the fact that devazepide crosses the blood brain barrier [248], also raises the possibility that devazepide induced c-Fos might be through acting on other brain regions and not by a direct effect on the SFO. As noted above, a possible explanation of this effect is that CCK_1 receptor stimulation might be activating neurons that send inhibitory signals to other neuronal populations. A situation like this has been previously shown in the amygdala, where Chung and Moore, using whole cell patch recordings, found that CCK-8 activates GABAergic interneurons thus increasing inhibitory transmission, however, in their study, this effect was CCK_2 receptor-mediated [249]. The induction of c-Fos by devazepide has been previously shown by Li and Rowland in the supraoptic nucleus, dorsomedial hypothalamus and the medial magnocellular subdivision of the hypothalamic paraventricular nucleus [129]. Pre-treatment with a combination of $CCKR_1$ and $CCKR_2$ antagonists resulted in an increase in c-Fos immunoreactivity similar to that

in the devazepide treated group. This finding rules out the possibility that blocking CCK_1 receptor alone might have caused basal circulating CCK to act on $CCKR_2$ receptors, hence activating the neurons.

To confirm and extend these findings, neuronal activation using the MAP kinase system was examined. Activation of the MAP kinase signaling cascade has been demonstrated to be associated with activation of the two subtypes of CCK receptors [250] and this pathway can be activated upstream of c-Fos expression or through a completely different signaling cascade from the one leading to c-Fos induction [251]. Previously, it has been shown that systemic administration of CCK leads to increased levels of phosphorylated form of ERKs, pERK1/2, in the NTS [169, 201]. Thus, by immunohistochemical localization of pERK, we studied the effect of systemic CCK on the number of pERK immunoreactive cells in the SFO. CCK-8 caused an increase in pERK immunoreactive neurons in the SFO and in the NTS, a region previously shown to display an increase in pERK immunoreactivity in response to CCK [169, 201]. The activation of the ERK pathway has been shown to be essential for the inhibitory action of CCK on food intake, since the use of an infusion of a MEK inhibitor into the fourth ventricle inhibited ERK cascade with subsequent attenuation of CCK-food suppressing effect [168]. The pattern of CCK induced pERK in the SFO was different from the c-Fos expression pattern, suggesting a different site of action. The CCK induced p-ERK positive SFO cells were more confined to the periphery of the SFO, which raises the possibility that CCK elicits differential effects in different population of SFO neurons. These results show that CCK induced c-Fos and p-ERK in the SFO is mediated through the CCK_2 receptor. The

importance of CCK_2 receptor in CCK mediated functions has been previously reported by a number of studies performed mostly in mice. For example, CCK_2 receptor deficient mice showed elevated body temperature, increased body weight and water consumption, suggesting a role for CCK_2 receptor in the processes regulating energy homeostasis [252]. Chen *et al*. also found increased body weight but reduced fat mass in mice lacking the CCK_2 receptor [253].

Taken together, the findings of this Chapter suggest that the SFO represents another CNS target for the actions of circulating CCK. We do not have evidence defining the role of CCK in the SFO, however, given the fact that CCK exerts a variety of physiological functions a number of possibilities exist. These include potential CCK actions in the SFO to modulate cardiovascular function that occur in parallel to the meal termination process [254]. CCK causes splanchnic vasodilatation that is followed by depressor and bradycardic effects [255]. CCK also inhibits renal sympathetic vasomotor nerve activity [256], resulting in increased blood flow to the gut and kidney to enhance the process of digestion and to deal with the additional fluid load following a meal. Thus the SFO may also represent an additional site in the CNS involved in the cardiovascular effects of CCK, a suggestion supported by the established roles of SFO in cardiovascular regulation [62, 257]. In addition, as mentioned earlier, lack of CCK_2 receptors resulted in increased water consumption in mice [252], raising the possibility that the SFO may act as a site mediating effects on drinking behavior. Finally, as mentioned earlier in this Chapter, although the well accepted roles of CCK to inhibit food intake and terminate meals are believed to be mainly mediated through the vagus nerve [231, 243, 244],

there are studies suggesting the presence of an extra-vagal site for CCK action [188-190] raising the possibility that circulating CCK may access extra-vagal sites of action such as the SFO and AP.

In conclusion, the results of this chapter show that CCK receptor activation leads to activation of the sensory CVO neurons in the AP and in the SFO. Using c-Fos and p-ERK as markers of activation, it was concluded that the CCK activation in the AP is mainly mediated by both $CCKR_1$ and $CCKR_2$ subtypes while CCK-induced activity in the SFO is a $CCKR_2$ dependent process. Given the importance of the vagus nerve in mediating CCK induced inhibition of food intake as well as activation of the brainstem, it is necessary to ask if CCK-induced action on the SFO is vagaly dependent or not. Moreover, the action of endogenously released forms of CCK on these sensory CVOs remains to be elucidated.

Chapter 3 Effect of vagotomy and endogenously released CCK on the activity of the subfornical organ and the area postrema

3.1 Introduction.

The results of Chapter 2 provided evidence for the response of the sensory CVOs namely the AP and the SFO to exogenous administration of CCK-8. Using c-Fos and p-ERK immunoreactivity as markers of neuronal activation, it was concluded that CCK results in activation of the AP and the SFO neurons. CCK-induced activation of the AP was mainly mediated by the CCK_1R subtype while SFO activation was CCK_2R-dependent. In this chapter, the contribution of the vagus nerve, as well as the effect of endogenously released CCK to the effect of CCK on the AP and the SFO will be examined. I will start with a brief background on the vagus nerve and its role in food intake regulation. Mechanisms and methods for induction of endogenous release of CCK will be discussed as well.

3.1.1 The vagus nerve.

The vagus is the cranial nerve that supplies the thoracic and abdominal viscera. In the thoracic region, the vagus nerve branches into the recurrent pharyngeal branch (innervating the larynx and upper esophagus) and the cervical cardiac branch (supplying the lower esophagus, trachea, bronchi, lungs and the heart). On the other hand, abdominal branches of the vagus include hepatic, gastric and celiac vagi [258]. The afferent fibers in the vagus nerve are much more numerous than the efferent ones present in an approximate ratio of 10 to 1, respectively. The cell bodies of the afferent vagal fibers originate in the nodose ganglion. The pseudounipolar primary vagal afferent

neurons run peripherally to the thoracic and abdominal viscera and project centrally to the brainstem to make synaptic connections with second order neurons located in the NTS [259].

The function of the afferent abdominal vagus is to sense and integrate various physiological stimuli within the GI tract [258]. This is achieved by the presence of a number of receptors that are responsive to different stimuli. Mechanoreceptors are one of these receptors that respond to change in tension and are mainly found in circular and longitudinal muscle layers [259]. Chemoreceptors which include sensors for nutrients or nutrient-related compounds comprise another type of receptors found in the vagal afferents. Examples include; nutrients such as glucose, amino acids and fatty acids as well as nutrient-related signals such as CCK, 5-HT, GLP-1 and somatostatin [258]. In addition to these, there is some evidence suggesting the presence of temperature sensors [260] and osmosensors [261] in some vagal afferent fibers. It should be noted that vagal afferents are mostly polymodal which means that a single fiber is capable of responding to more than one type of stimulus.

The vagus nerve is considered a very important component in the gut-brain axis in the control of ingestive behaviors. As food is consumed, vagal afferents sensitive to mechanical deformation detect its presence and gastric distension takes place leading to activation of vagal mechanoreceptors within the muscular layers of the stomach. When the food reaches the intestine, vagal afferents, found in close proximity to CCK-secreting enteroendocrine cells, are activated upon the release of CCK [262]. Moving down the gastric tract, the vagal afferents sense and interact with other hormones like

GLP-1 and PYY [263, 264]. All these inputs are then relayed and integrated in the NTS, AP, and dorsal motor nucleus of the vagus (DMV) [265, 266] to initiate the process of satiation and termination of feeding. To study the role of the vagal afferents in the negative feedback control of feeding, total subdiaphragmatic vagotomy has been extensively used. This surgical procedure is found to result in a number of effects, for example; blocks the ability of gastric distension to inhibit feeding [267], attenuates nutrient induced inhibition of feeding [268, 269] and blocks the satiety effect of exogenous CCK [185, 186]. To study the contribution of specific branches of the vagus, selective vagotomies are performed such as gastric branch vagotomy [270] and hepatic branch vagotomy [271]. In addition to surgical dissection of the vagus, vagal capsaicin application is also used to study the contribution of the vagus nerve in visceral sensation [169, 188, 272]. Capsaicin is a neurtotoxin that destroys small unmyelinated primary sensory neurons leading to degeneration of vagal sensory fibers [273].

3.1.2 Endogenous release of CCK

As mentioned in Chapter 2, CCK is a hormone secreted from intestinal I cells in response to feeding. In this chapter, I will discuss the mechanisms by which endogenous CCK is released and how can we induce its release for the purpose of studying the effects of endogenous CCK on neuronal activation in the brain. The normal physiological stimulus for CCK release is feeding. A meal is usually composed of three main components; proteins, fats and carbohydrates. The two main meal elements that have been found to stimulate the release of CCK are amino acids from proteins and fatty acids from fats with carbohydrate being a poor stimulant of CCK release [274]. A rise in

plasma CCK concentration is usually used as an indicator for the release of CCK, however, it is important to note that in certain circumstances a local release of CCK can activate the vagal afferents without causing a significant effect on plasma levels [43]. Basal plasma CCK level is about 1 pM and rises to 5-8 pM following a meal [229].

Earlier studies identified intestinal fats as stimulator of CCK release [275, 276]. Later on it was concluded that only long chain fatty acids are responsible for CCK release [277]. Recently, using a transgenic mouse model that expresses green fluorescent protein under the control of CCK promoter to identify and isolate intestinal I cells, it has been found that a G-protein coupled receptor namely GPR40 is expressed in these cells. This receptor interacts with long chain fatty acids and causes a rise in intracellular Ca^{++} leading to the release of CCK [278]. Similarly, it was found that amino acids interact with a Ca^{++} sensitive receptor (CaSR) which has been found to be expressed in intestinal I cells resulting in increased intracellular Ca^{++} with subsequent release of CCK [279-281].

In addition to fatty acids and amino acids, special CCK releasing factors have been identified. These factors are inactivated in the presence of the trypsin enzyme leading to reduction of CCK release. So far, three CCK releasing factors have been identified; monitor peptide, luminal CCK-releasing factor (LCRF) and diazepam-binding inhibitor (DBI) [282, 283]. These factors act in a similar fashion to activate CCK releasing cells however, they differ in their origin; the monitor peptide is of pancreatic origin however, LCRF and DBI are released from the intestine [282, 283]. These peptides are sensitive to trypsin activity which explains why ingestion of proteins that compete for trypsin or administration of trypsin inhibitors causes the release of CCK [282, 284].

Therefore, feeding as well as different types of trypsin inhibitors are both described in the literature as stimulators of the endogenous release of CCK. For example, Reidelberger used liquid mashed diet to assess the contribution of the vagus to endogenous CCK action [190]. Intake of 20% sucrose solution was also used to study the contribution of CCK receptors to the action of endogenous CCK [285]. Intestinal nutrient infusion [286, 287] as well as synthetic trypsin inhibitors such as camostat [288] or natural occurring ones (i.e. Soybean trypsin inhibitor)[289, 290] have been used to induce the release of CCK.

Besides intestinal I cells as a source of peripheral CCK, CCK is released from neurons in the brain and is considered one of the most abundant central neurotransmitters [291, 292]. Similar to intestinal CCK, brain CCK was also found to be released in response to feeding. For example, increased hypothalamic and dorsal motor nucleus CCK was observed after a meal which suggests the function of central CCK as a satiety signal [293, 294]. Schick and colleges found that electrical stimulation of the vagal afferents results in hypothalamic release of CCK and intragastric loads of nutrients or water also increase hypothalamic CCK in a vagally dependent manner [295].

3.1.3 Hypothesis and Aim.

In Chapter 2, I showed that the intraperitoneal administration of exogenous CCK-8 lead to increased c-Fos as well as pERK immunoreactivity within the SFO, indicating that neurons in this region were activated. By using selective CCK_1 and CCK_2 receptor antagonists, it was concluded that this effect is mainly mediated via a $CCKR_2$ pathway. Accordingly, my hypotheses in this chapter are:

1- The effect of exogenous CCK on the SFO is not mediated by the vagus nerve.

2- Endogenously released CCK will activate SFO neurons through $CCKR_2$.

Thus, the aims of this chapter are to:

1. Study the role of the vagus nerve in mediating the action of CCK on the SFO.

2. Study the effect of endogenous CCK released by a meal or a soybean trypsin inhibitor on the activity of the SFO as well as selected regions in the brainstem.

3. Study the role of $CCKR_1$ and $CCKR_2$ in mediating the effects of endogenous CCK on activation of the SFO and the brainstem.

3.2 Materials and Methods.

3.2.1 Animals.

Male Sprague-Dawley rats (130-180 gm) or male Sprague Dawley (SD) rats with or without a complete subdiaphragmatic truncal vagotomy (100-120gm) were purchased from Charles River (Montreal, Quebec, Canada). Upon arrival, they were housed under specific pathogen free conditions on a reversed 12-hour reverse light-dark cycle with free access to food and water for one week before the experiment.

3.2.2 Compounds.

Cholecystokinin (sulphated CCK-8; catalog no. 069-03) was purchased from Phoenix® (Phoenix Pharmaceuticals, Burlingame, CA, USA). Devazepide and L-365.260 were purchased from Tocris® (Tocris Bioscience, Minneapolis, MN, USA) and Soybean trypsin inhibitor was purchased from Calbiochem® (EMD Millipore, MA, USA; catalogue no. 65035).

3.2.3 Verification of subdiaphragmatic vagotomy.

Upon arrival from Charles River, both vagotomized and sham-operated rats were placed on Ensure© Liquid diet and allowed to recover for 10 days. Body weight as well as food intake was measured daily. To verify the completeness of the vagotomy, the effect of an intraperitoneal injection of CCK-8 [296] on food intake was measured. One day before the experiment, vagotomized rats (Vgx) as well as sham-operated (sham) rats were fasted for 18 hours. On the day of the experiment, Vgx and sham rats were divided into two groups each. Each group received either vehicle or CCK-8 (i.p.). Five

minutes later, each animal was provided a pre-measured amount of Ensure liquid diet and Ensure intake was measured at 30 and 60 minutes intervals. Two days later, the same experiment was repeated in a cross over design to ensure that all rats are tested for CCK sensitivity.

3.2.4 Effect of i.p.CCK-8 on food intake in sham operated and vagotomized rats.

Intraperitoneal administration of CCK-8 (16 μg/kg) was tested for its effect on reducing food intake in both sham and vagotomized rats following the same procedures described in Chapter 2.

3.2.5 Effect of i.p.CCK-8 on c-Fos immunoreactivity in sham operated and vagotomized rats.

Intraperitoneal administration of CCK-8 (16 μg/kg) was used to study its effect on c-Fos immunoreactivity in the AP and NTS, as well as in the SFO, in both sham and Vgx rats (n=4/group) following the same procedures outlined in Chapter 2. For ease of comparison, the numbers of c-Fos immunoreactive neurons in CCK-treated sham and Vgx rats were expressed as a percentage increase of vehicle treated respective controls.

3.2.6 Effect of Soybean trypsin inhibitor on food intake.

In order to study the effect of SBTI in reducing food intake, rats were divided into two groups; control: receiving an oral gavage of 2 ml distilled water or SBTI (100 mg/kg p.o). The dose of SBTI was chosen based on previous literature [290, 297]. A pre-weighed amount of rat chow was offered to each rat and food was measured every 30 minutes for 4 hours.

3.2.7 Effect of Soybean trypsin inhibitor on c-Fos immunoreactivity.

The same immunohistochemical procedure was followed for studying the effect of SBTI on c-Fos immunoreactivity, however, we modified the time of sacrifice after orally giving SBTI to be 120 minutes instead of 90 minutes after intraperitoneal injections based on the results of the feeding studies described above.

3.2.8 Liquid Ensure© feeding schedule.

To study the effect of a meal on activation of the SFO, AP and NTS, animals were put on a daily feeding schedule of drinking an Ensure© meal for one hour just at the beginning of their dark cycle. The rats were then offered rat chow for the rest of the day. The amount of Ensure consumed was measured at 30 and 60 minutes. This procedure was done until a stable baseline is reached among all rats. It was noticed that rats will consume approximately 20 ml of Ensure in the first 20 minutes and accordingly this amount was chosen to be used as a meal for all c-Fos studies.

3.2.9 Effect of Ensure© on c-Fos immunoreactivity.

Rats adapted to daily handling and injection with saline for at least 5 days were fasted overnight with free access to water. At the day of the experiment, 4 rats were given access to a 20 ml liquid Ensure meal to be fully consumed in 20 minutes while the other 4 rats were left fasted to serve as a fasting control. The food containers were then removed. Ninety minutes after the initial start of feeding, animals were anesthetized with sodium pentobarbital (50 mg/kg), perfused intracardially with 200 ml saline followed by 300 ml 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.3).

Brains were dissected and processed for c-Fos immunohistochemistry as described in chapter 2.

3.2.10 Effect of CCK antagonists on Ensure induced c-Fos immunoreactivity.

Rats adapted to daily handling and injection with saline for at least 5 days were fasted overnight. At the day of the experiment, rats were divided into 4 groups; these groups received vehicle, devazepide (600 μg/kg), L-365.260 (100 μg/kg) or devazepide + L-365.260 thirty minutes prior to access to a 20 ml liquid Ensure meal to be fully consumed in 20 minutes. The food containers were then removed. Ninety minutes after the initial start of feeding, animals were anesthetized with sodium pentobarbital (50 mg/kg), perfused intracardially with 200 ml saline followed by 300 ml 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.3). Brains were then dissected and processed as mentioned before.

3.2.11 Statistics.

All statistical analyses were performed using GraphPad Prism (version 6.0; San Diego, CA, USA). When comparing two groups, data were analyzed using Student's t test. Analysis of Variance (ANOVA) test was used for multiple comparisons followed by Bonferroni post tests.

3.3 Results

3.3.1 Effect of vagotomy on CCK induced c-Fos immunoreactivity in NTS, AP and SFO.

3.3.1.1 Verification of subdiaphragmatic vagotomy:

Rats subjected to subdiaphragmatic vagotomy showed a reduced weight gain compared to sham-operated rats. As shown in Figure 3.1, Vgx rats showed reduced body weight gain (A) and liquid diet intake (B) compared to sham-operated controls. One week after the surgery, the body weight of vagotomized rats was significantly lower than sham-operated rats (235.6 gm ± 2.3, n=8 for sham versus 169.9 gm ± 6.5; n=10 for Vgx; Figure 3.2 A). The loss of body weight helped confirm the success of the vagotomy as previously described [298, 299]. Moreover, to assess the completeness of the subdiaphragmatic vagotomy, food intake in response to intraperitoneal injection of CCK (16 µg/kg) was measured. This dose was relatively higher than the doses usually used to test the effectiveness of vagotomy [185, 186, 299, 300]. However, this dose is consistent with the dose used in Chapter 2 examining the effect of CCK on the activity of the SFO [223]. Despite using a higher dose of CCK, all rats subjected to vagotomy failed to show a reduction of food intake in response to CCK. On the other hand, all sham operated rats showed a significant reduction of food intake in response to CCK (7.4 ml ± 0.1 for vehicle treated versus 5.0ml ± 0.6 for CCK treated sham rats, Figure 3.2 B). Furthermore, at the time of perfusion, we observed that the Vgx rats had enlarged stomachs (Figure 3.3) compared to sham-operated rats indicative of impaired gastric motility associated with vagotomy [301].

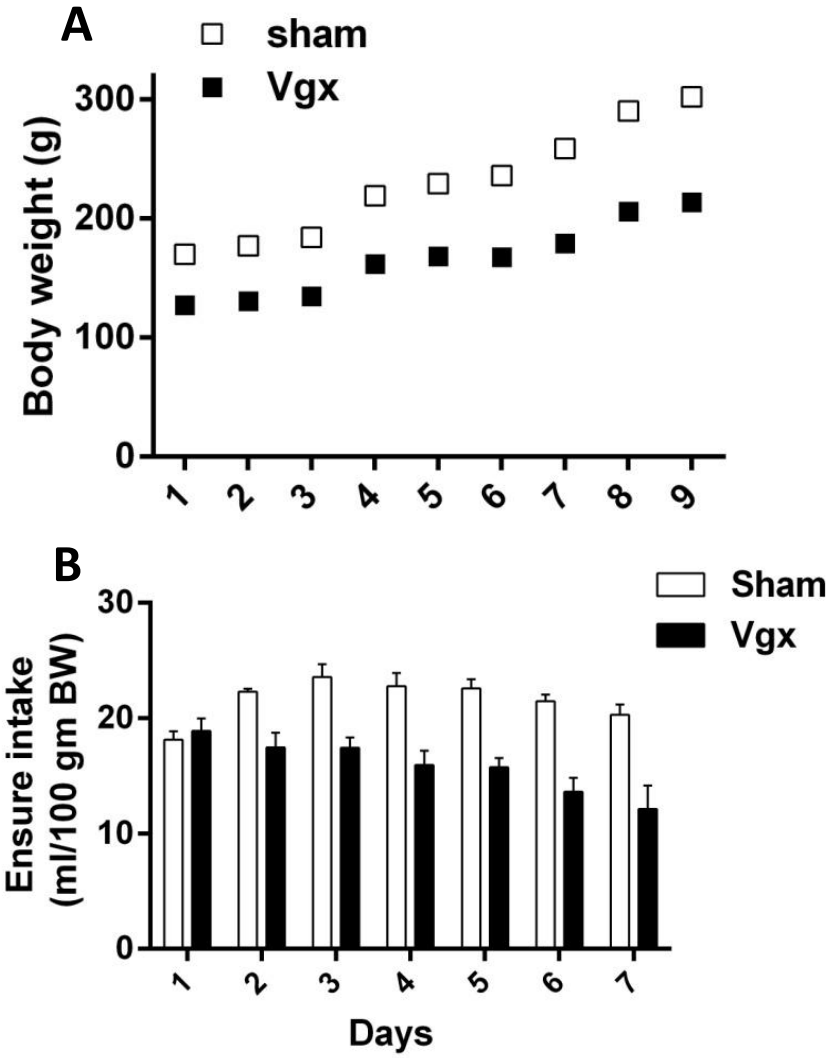


***Figure 3.1*: Effect of vagotomy on body *weight* gain and food intake.**

A: Line graph showing body weight gain of sham-operated and vagotomized rats.

B: Bar graph showing daily 5 hours food intake/100 gm body weight of sham-operated and vagotomized rats. Vgx: vagotomized rats.

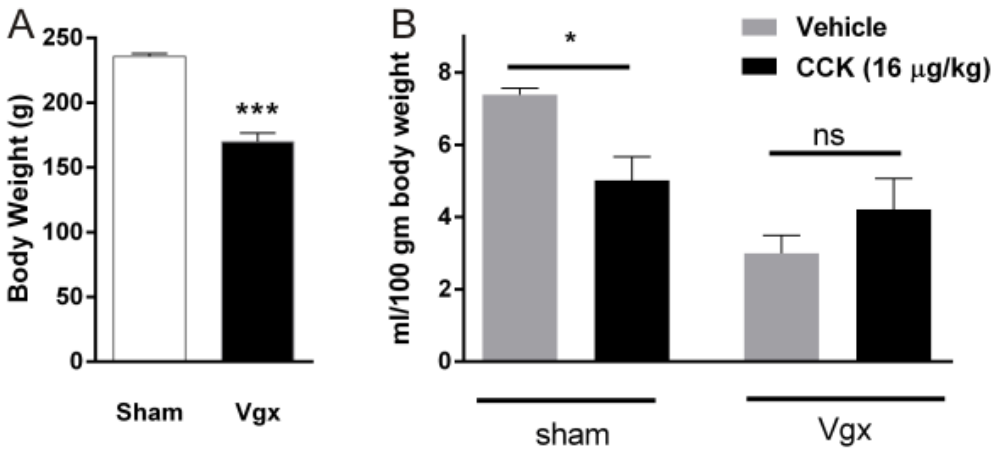


Figure 3.2: Effect of vagotomy on body weight and CCK action.

A: Bar graphs showing body weight difference between sham-operated and vagotomized rats.

B: Effect of ip CCK on food intake in sham and vagotomized rats. Data are presented as the mean ± S.E.M, *$p<0.05$, *** $p<0.001$ represent a significant difference from the respective control. ns: non-significance difference, Vgx: vagotomized rats.

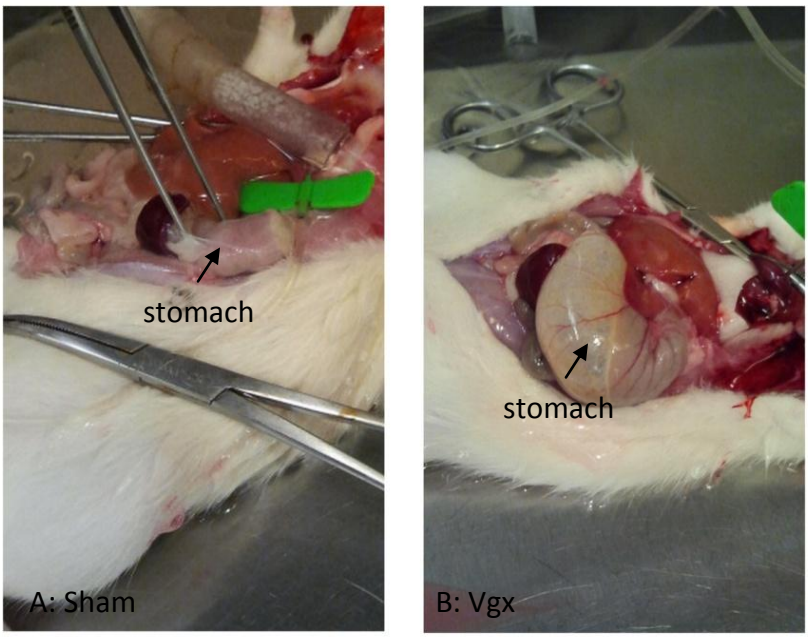


***Figure 3.3*: Comparison between the stomachs of sham-operated and vagotomized rats.**

Representative photos taken during perfusion showing stomach distension in vagotomized rat (B) compared to sham-operated rat (A).

3.3.1.2 Effect of vagotomy on CCK-induced c-Fos immunoreactivity in the SFO.

As shown in Figure 3.4 A and B, vagotomy resulted in a significant reduction in the number of c-Fos immunoreactive neurons in response to CCK in the AP and NTS (149.3% ± 50.7 for AP and 135.7% ± 8.9 for NTS) compared to sham-operated rats (435.9% ± 31.12 for AP and 477.4% ± 30.0 for NTS) expressed as % increase from vehicle treated Vgx and sham rats, respectively. It should be noted that relatively higher c-Fos immunoreactivity in vehicle treated Vgx rats compared to sham-operated rats in the brainstem areas NTS and AP was observed.

In the SFO, vagotomy had no significant effect difference on CCK-induced c-Fos immunoreactivity compared to the sham-operated group as illustrated in Figure 3.4 A and C. The pattern of c-Fos expression in response to CCK in Vgx or sham rats was more prominent in the core of the central region of the SFO (bregma -0.92 to -1.3) consistent with that shown before with i.p. CCK in unoperated rats in Chapter 2.

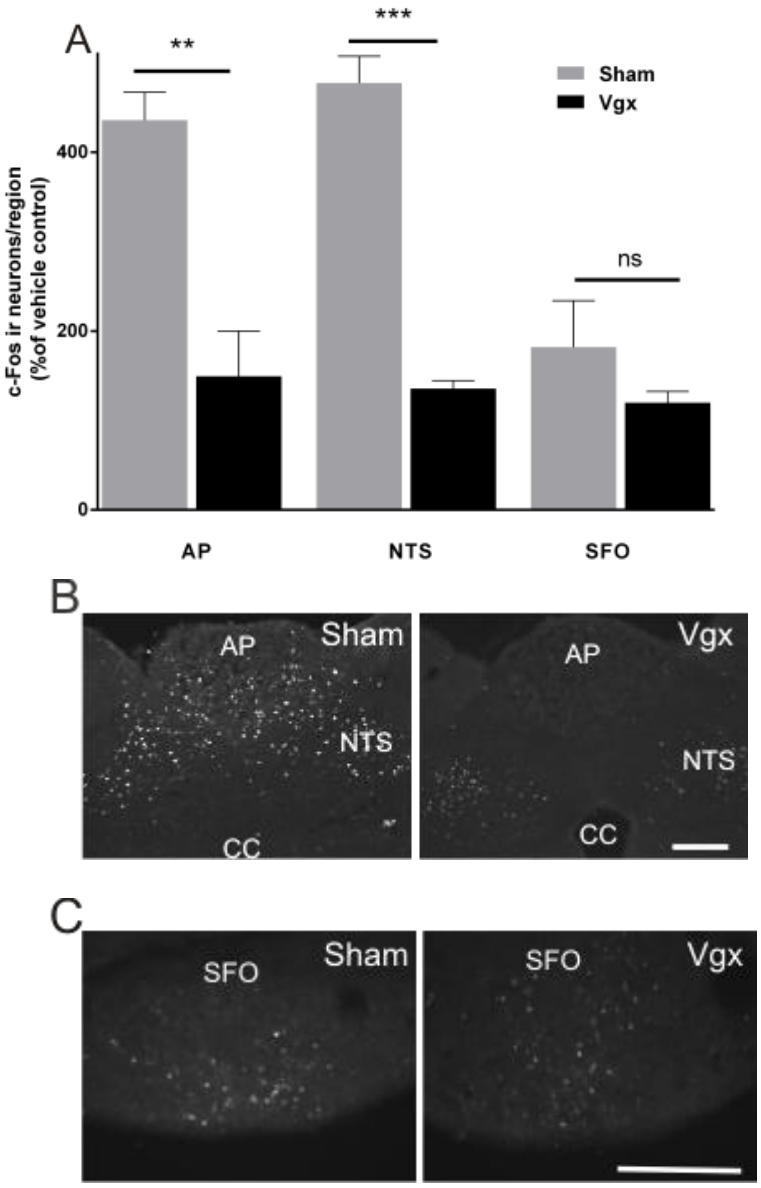


***Figure 3.4*: Effect of i.p CCK on c-Fos immunoreactivity in the brainstem and SFO.**

A: Bar graphs comparing c-Fos immunoreactity expressed as percentage increase from control in sham-operated versus vagotomized rats in the AP, NTS and the SFO (A). Data are presented as the mean ± S.E.M, ** $p<0.01$, *** $p<0.001$ represent a significant difference from the respective control.

Representative immunofluorescence micrographs are shown in B and C. c-Fos immunoreactivity was lower in vagotomized animals injected intraperitoneally with CCK in the AP and the NTS (B), while no significant difference was observed in c-Fos immunoreactivity in the SFO in Sham and vagotomized rats after injection of CCK. NTS: nucleus of the solitary tract, AP: area postrema, cc: central canal, SFO: subfornical organ. Scale bar = 100 µm.

3.3.2 Effect of endogenously released CCK on the activity of the SFO.

3.3.2.1 Effect of Soybean trypsin inhibitor on food intake.

Oral administration of SBTI in a dose of 100 mg did not result in a significant reduction of food intake at 30 minutes; however it caused a significant reduction in food intake 60 minutes after administration (Figure 3.5).

3.3.2.2 Effect of Soybean trypsin inhibitor on c-Fos immunoreactivity in NTS, AP and SFO.

As illustrated in Figure 3.6 A and B, oral administration of SBTI resulted in a significant increase in the number of c-Fos immunoreactive cells in the AP (25.3 cells ± 2.8 for SBTI versus 10.0 cells ± 2.0 for control) and in the NTS (61.0 ± 6.5 for SBTI versus 21.3 ± 3.1 for control) compared to non treated control. It should be noted that although gavaging SBTI resulted in a significant increase in the c-Fos count in the AP and in the NTS, the numbers of c-Fos positive neurons was not as high as the numbers observed after an Ensure meal (62.8 cells ± 12.8 for AP and 180.0 cells ± 13.4 in the NTS). Conversely, in the SFO, SBTI failed to induce a significant change in c-Fos immunoreactivity compared to control rats (Figure 3.6 A and C).

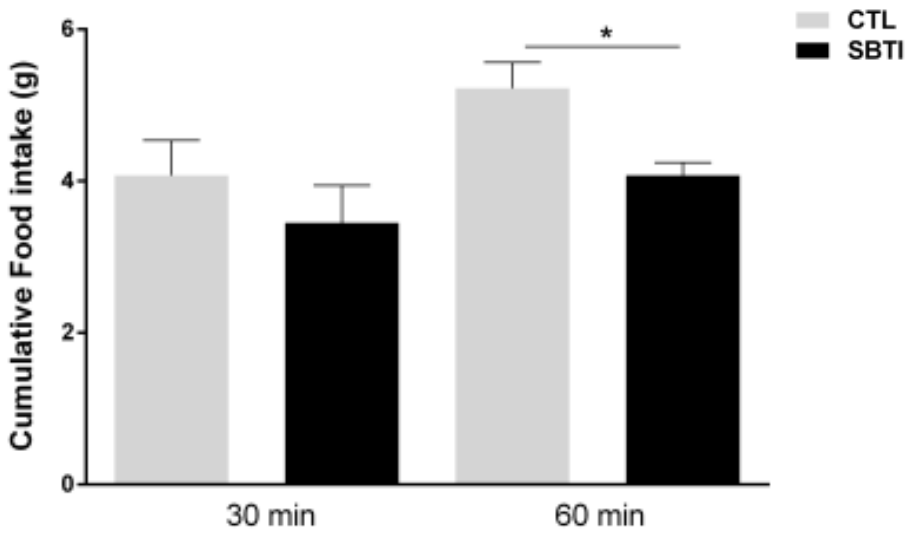


***Figure 3.5*: Effect of SBTI on food intake.**

Bar graph presenting the effect of SBTI on food intake after thirty and sixty min of intake. Data are presented as the mean ± S.E.M, * $p<0.05$, represents a significant difference from the respective control.

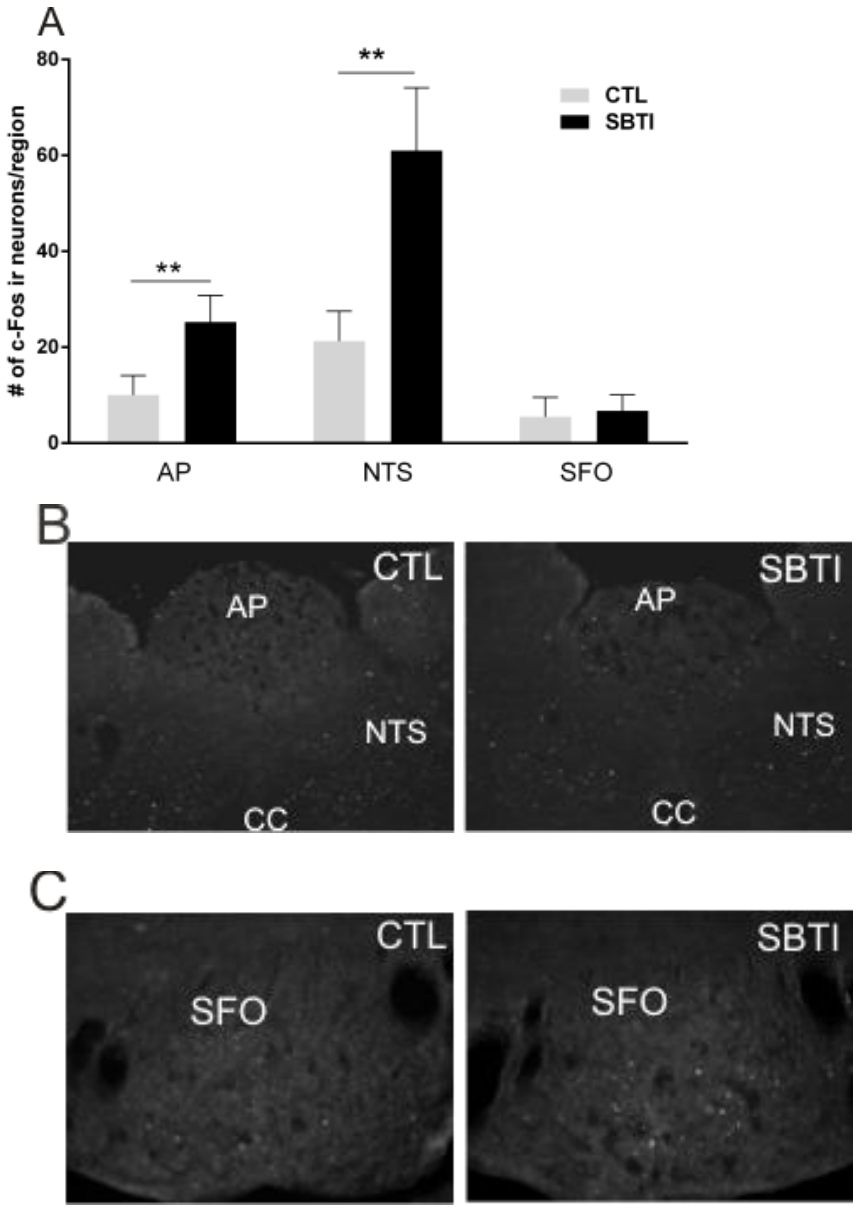


Figure 3.6: **Effect of SBTI on c-Fos immunoreactivity.**

Bar graphs comparing the numbers of c-Fos immunoreactive cells in the AP, NTS and the SFO (A). Data are presented as the mean ± S.E.M, ** $p<0.01$ represents a significant difference from the respective control. Representative immunofluorescence micrographs are shown in B and C. NTS: nucleus of the solitary tract, AP: area postrema, cc: central canal, SFO: subfornical organ. Scale bar = 100 µm.

3.3.2.3 Effect of Ensure on c-Fos immunoreactivity in the brainstem and the subfornical organ.

As shown in Figure 3.7 (A and C), feeding caused a significant increase in c-Fos immunoreactive neurons in the AP and in the NTS; 84.7 ± 1.5 and 169.3 ± 10.7 compared to control fasted rats; 11.8 ± 2.2 and 36.5 ± 7.0, respectively. Similarly, feeding liquid Ensure meal resulted in a significant increase in the number of c-Fos positive neurons in the SFO compared to fasted controls (10.0 cells ± 1.0 for fasted versus 28.8 cells ± 2.9 for fed rats, Figure 3.7 B and D).

It is important to point out the differences observed in the pattern of c-Fos expression previously shown in Chapter 2 following i.p. CCK administration from the one shown here induced by a meal. Figure 3.8 A and B shows a diagram illustrating the difference in c-Fos distribution in response to i.p. CCK and Ensure. As shown in this Figure, the c-Fos positive neurons in response to CCK tend to be localized in the core region of the SFO. The core region of the SFO is more vascularized with abundant fenestrated capillaries, making this region potentially more accessible to circulating agents [246]. After feeding, the c-Fos positive cells are distributed between the inner core and outer shell region of the SFO raising the possibility of the contribution of different stimuli in the SFO activation following a meal.

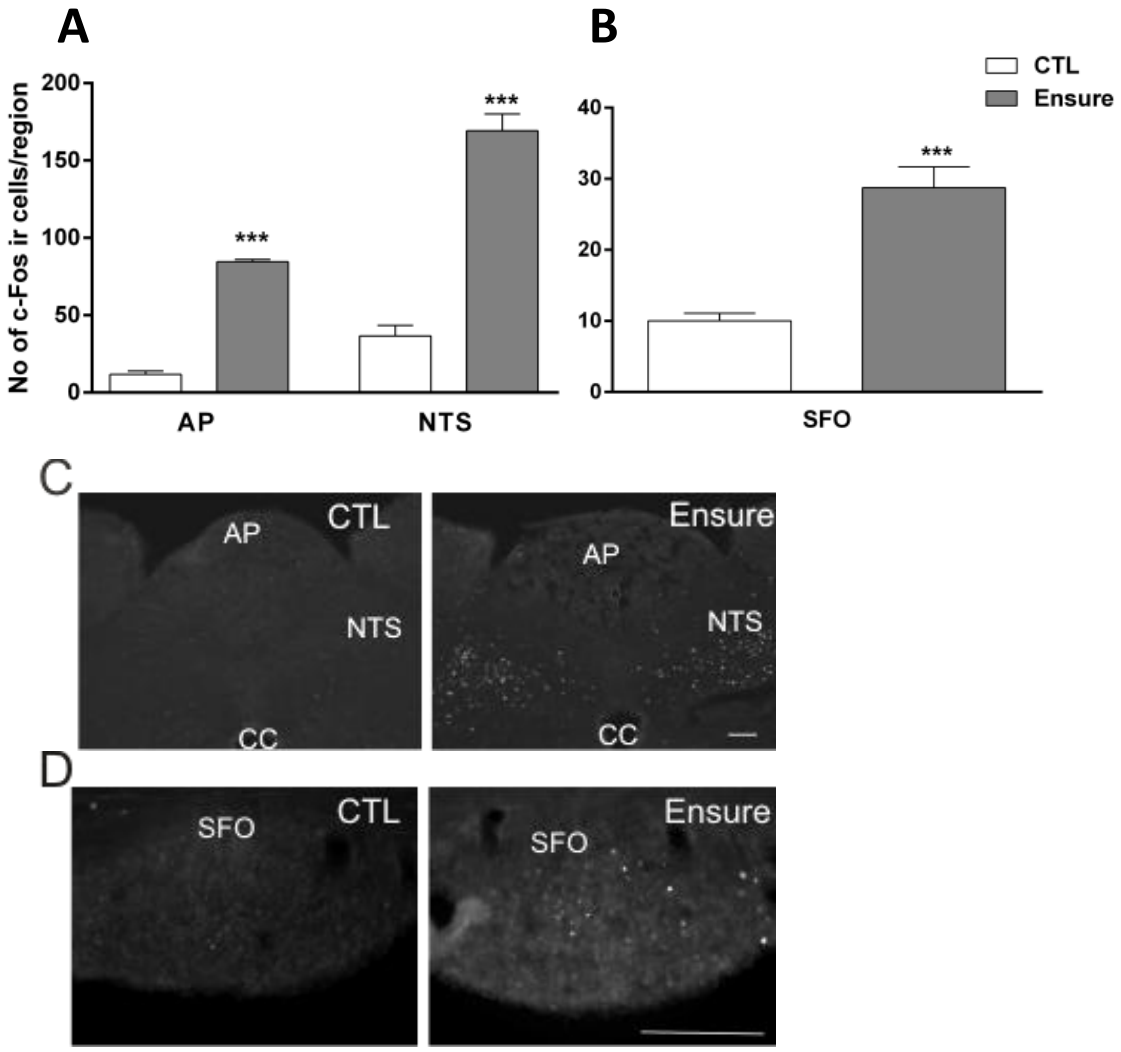


***Figure 3.7*: Effect of liquid Ensure© meal on c-Fos in the brainstem and in the SFO.**

Bar graphs comparing the number of c-Fos immunoreactive cells in response to Ensure meal in the AP and NTS (A) and in the SFO (B). Data are presented as the mean ± S.E.M, *** $p<0.001$ represents a significant difference from the respective control.

Representative immunofluorescence micrographs are shown in C and D. c-Fos immunoreactivity in response to Ensure was higher than control animals (CTL) in the AP, NTS (C) as well as in the SFO (D). NTS: nucleus of the solitary tract, AP: area postrema, cc: central canal, SFO: subfornical organ. Scale bar = 100 µm.

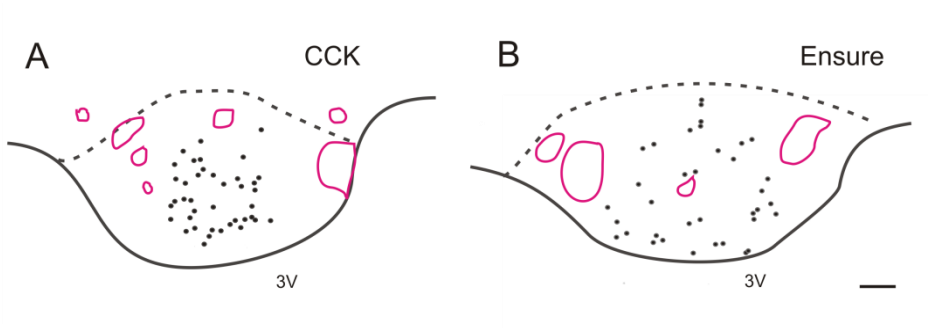


Figure 3.8: Comparison between c-Fos ir in response to ip CCK and Ensure.

A diagram comparing the distribution of c-Fos immunoreactive cells in the SFO in response to ip CCK (A) and feeding a liquid Ensure meal (B). 3V: third ventricle, red outlined structures represent blood vessels. Scale bar = 100 µm.

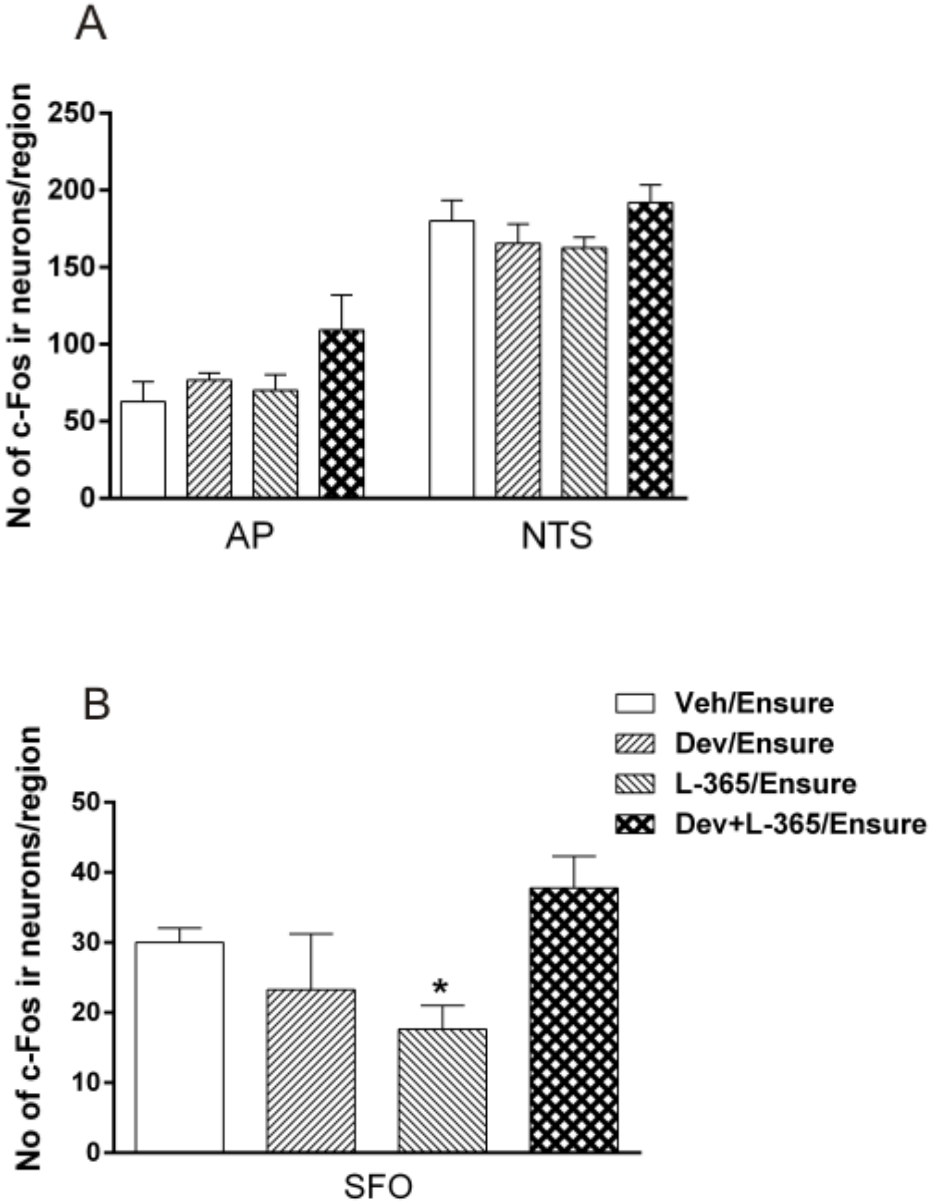
3.3.2.4 Effect of CCK antagonists on Ensure induced c-Fos immunoreactivity in the NTS, AP and SFO.

The next step was to ask if a meal induced c-Fos immunoreactivity in the brainstem regions AP and NTS and in the SFO is mediated by CCK. I studied this by testing the effects of pretreatment with a CCK_1 receptor antagonist (devazepide), a CCK_2 receptor antagonist (L-365.260), or a combination of both compounds on c-Fos immunoreactivity induced by feeding. As shown in Figure 3.9, pretreatment with devazepide (600 µg/kg) prior to access to Ensure meal did not significantly affect meal-induced increase in c-Fos immunoreactive neurons in the AP (Ensure; 62.8 ± 12.8 versus devazepide/Ensure; 76.8 ± 4.7), the NTS at the level of the AP (Ensure; 180.0 ± 13.4 versus devazepide/Ensure; 165.5 ± 12.6); or the SFO (Ensure; 30.0 ± 2.0 versus devazepide/Ensure; 23.3 ± 8.0; Figure 3.9 B and C). These results were not surprising as it has been shown before that meal induced c-Fos in the brainstem is not dependent on CCK_1 receptor [112] .

Pretreatment with the CCK_2 receptor antagonist; L-365.260 (100µg/kg) did not affect the meal-induced increase in the number of c-Fos immunoreactive neurons in the AP and the NTS Figure 3.9 A. However, as shown in Figure 3.9 B and C, L-365.260 significantly attenuated Ensure-induced c-Fos immunoreactivity in the SFO (L-365.260/Ensure; 17.7 cells ± 3.3, versus Ensure; 30.0 cells ± 2.0).

Combination of CCK_1 and CCK_2 receptor antagonists with a meal showed no significant difference in c-Fos expression in the AP and in the NTS as well as in the SFO, compared to the effect of feeding alone (Figure 3.9 A-C). The observed effect of

combining the two antagonists was not surprising because in chapter 2, it was shown that devazepide alone induces c-Fos in the SFO and combining both antagonists did not block the effect of systemic CCK on the SFO.


A
No of c-Fos ir neurons/region
250
200
150
100
50
0
AP
NTS
B
No of c-Fos ir neurons/region
50
40
30
20
10
0
*
SFO
Veh/Ensure
Dev/Ensure
L-365/Ensure
Dev+L-365/Ensure

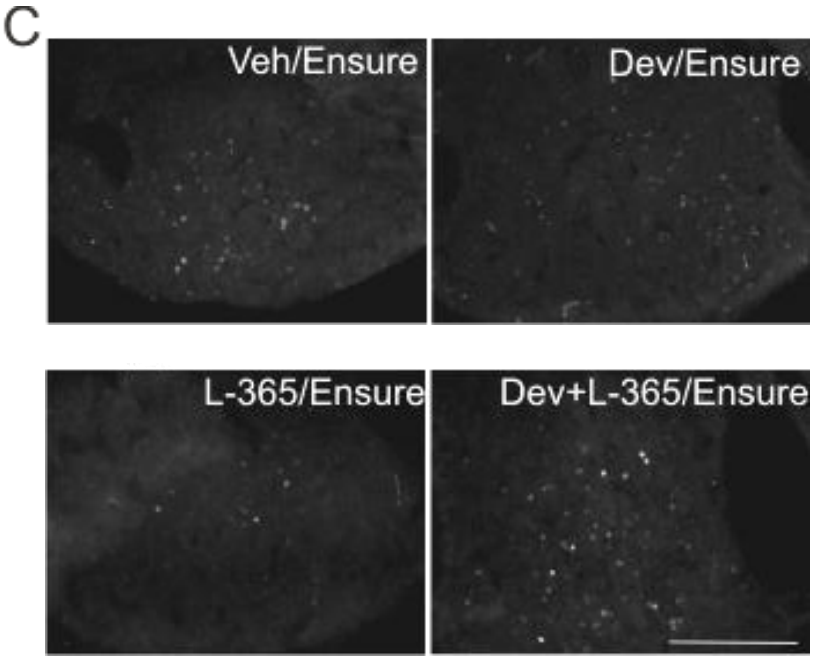


Figure 3.9: **Effect of CCK antagonists on Ensure induced c-Fos immunoreactivity.**

Bar graphs comparing the number of c-Fos immunoreactive cells in four different groups pre-treated with Vehicle (Veh/Ensure), devazepide (600 µg/kg; Dev/Ensure), L-365.260 (100 µg/kg; L-365/Ensure) and a combination of both (Dev+L-365/Ensure) in the AP/ NTS (A) and in the SFO (B). Data are presented as the mean ± S.E.M, *$p<0.05$ represents a significant difference from the respective control.

Representative c-Fos immunofluorescence micrographs in the SFO from the four different groups are shown in C. Scale bar = 100 µm.

3.4 Discussion.

Following the initial observation of the effect of systemic CCK injection on the activity of the SFO (Chapter2), the studies presented in this Chapter aim at gaining more understanding about the role of the vagus nerve and endogenous CCK in CCK-induced SFO activation. First, using rats subjected to subdiaphragmatic vagotomy, it was found that the effect of ip CCK on c-Fos immunoreactivity in the SFO is not dependent on an intact vagus, in contrast to the well-established effect of CCK on the brainstem region, NTS [302-304]. The next step was to study the contribution of endogenous CCK to SFO activation; two approaches were utilized to induce the release of CCK; a trypsin inhibitor and a meal. It was found that inducing a local release of CCK by soy bean trypsin inhibitor did not cause the induction of c-Fos immunoreactivity in the SFO, while using a liquid Ensure meal resulted in a significant increase in c-Fos in the SFO, as well as the brainstem regions, AP and NTS. The contribution of CCK in meal induced activation of the SFO, using selective CCK_1 and CCK_2 receptor antagonists was studied and it was found that meal-induced SFO activation is partly mediated through the CCK_2 receptor. Collectively, these studies extend the results in Chapter 2 and show that under physiological conditions, the SFO is activated by CCK.

Total subdiaphragmatic vagotomy, the surgical dissection of the subdiaphragmatic branches of the vagus nerve, has been extensively utilized to determine the role of vagal afferents in viscerosensory signaling [204, 305-309]. Accordingly, rats subjected to subdiaphragmatic vagotomy (Vgx) were used to study the contribution of the vagus nerve in CCK-mediated activation of the SFO. In this study, rats

subjected to Vgx as well as sham-operated controls were kept on Ensure liquid diet. It has been shown that substitution of normal rat pellets with liquid diet partially ameliorates the post-vagotomy syndrome of hypophagia and reduces weight loss as well as reducing mortality rate after surgery [310]. The inhibitory effect of CCK on food intake was abolished in Vgx rats. This effect has been shown before either by using surgical vagotomy [185, 186] or by applying capsaicin to desensitize vagal afferents [184, 187]. Joyner *et al.* found that vagotomy abolishes the effect of 1 and 4 μg of CCK and partially reduced the effect of 8 μg [186]. On the other hand, in the current study, the effect of a much higher dose of CCK-8 (16 μg/kg) was completely abolished as a result of Vgx. The lack of response to a high dose of CCK in my study could be attributed to the relatively shorter time allowed for recovery of Vgx rats (2 weeks), compared to 8-10 weeks in the study by Joyner *et al.* [186]. The findings by Peters *et al.* [311] provide further support for the difference explained above. It was found that Vgx triggers transient significant withdrawal of central vagal afferent terminals in the NTS 10 days following Vgx with gradual restoration of the terminals that is complete at 60 days after surgery [311].

The dose of CCK used did not produce a significant increase in c-Fos immunoreactivity in the the AP and the NTS in Vgx compared to sham control. This has been previously shown by other groups [243, 302, 312]. However, vagotomy did not cause a reduction of CCK-mediated c-Fos expression in the SFO. These results suggest that the effect of CCK on the SFO might be mediated through a direct interaction between circulating CCK and the SFO which is characterized by a leaky blood brain

barrier or by acting on neurons that project to the SFO from other brain regions. This also raises the possibility that CCK in the SFO is mediating other effects other than to influence food intake which shows dependence on an intact vagus. More studies are needed to elucidate the physiological output of CCK-mediated activation of the SFO.

The next step was to investigate the effect of endogenously released CCK on the activity of the SFO. Feeding as well as different types of trypsin inhibitors are common tools used as stimulators of the release of endogenous CCK [190, 285, 288-290]. Two different approaches to induce the release of CCK were utilized; by oral soy bean trypsin inhibitor and by feeding an Ensure liquid meal, to test the effect of these treatments on c-Fos immunoreactivity in the SFO.

Oral administration of soy bean trypsin inhibitor resulted in a delayed onset reduction of food intake when compared to i.p. CCK. SBTI caused a reduction in food intake sixty minutes after oral gavage while CCK induces a reduction in food intake thirty minutes after intake. The effect of trypsin inhibitors on food intake has been controversial depending on the type of inhibitor used and the experimental setup. For example, Smith *et al.,* found no effect of SBTI on real or sham feeding but they only studied the effect at 30 minutes after administration [313]. On the other hand, Garlicki *et al.* showed a reduction in food intake following oral trypsin inhibitor; however in this study they gave their trypsin inhibitor 2 hours before the start of food intake measurements [314]. We studied animals 2 hours after SBTI and focused on whether SBTI caused neuronal activation.

Similar to previous reports, a significant increase in c-Fos immunoreactive cells in the AP and the NTS in response to SBTI were found [288, 315]. However, no effect was observed in the SFO. This can be explained in light of the postulation made by Raboin *et al.* (illustrated in Figure 3.10) that trypsin inhibitors cause a local release of CCK that activates the submucosal plexus and cause the activation of vagal afferents leading to c-Fos expression in the dorsal vagal complex of the brainstem. However, trypsin inhibitor releases an insufficient amount of CCK to increase circulating CCK that reaches the SFO to cause its activation [288]. However, it is important to point out that other studies found increased circulating CCK in response to trypsin inhibitors comparable to the increase followed by a meal [316-318]. Thus, further studies are needed to understand the lack of effect of SBTI on the activity of the SFO.

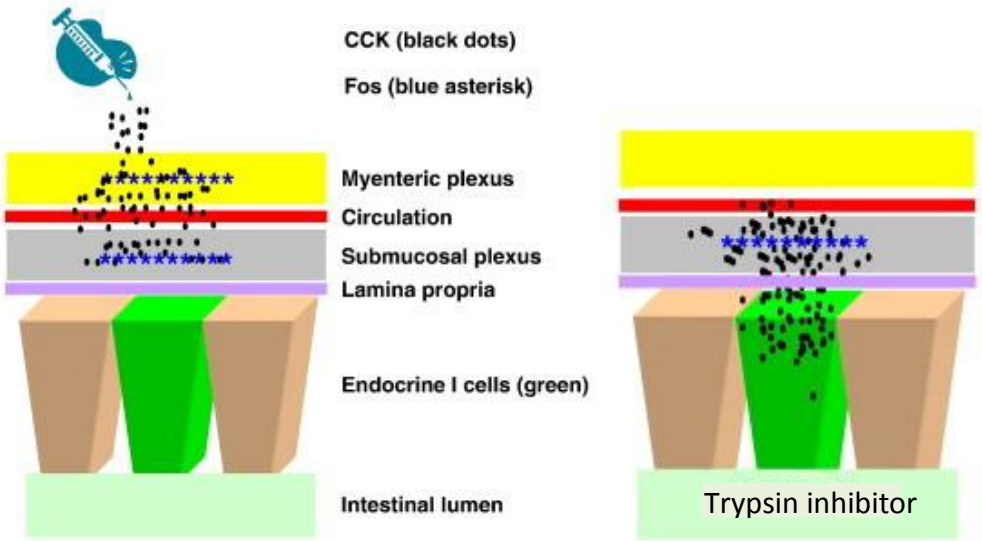


Figure 3.10: Illustration showing the effect of exogenous and endogenous CCK on CCK diffusion.

The diagram shows that exogenous CCK. Is injected i.p. diffuses by the peritoneal fluid, through the outermost serosal layer of the gut wall and activates the myenteric plexus, then diffuses further to activate the submucosal plexus (left panel). In the right panel, endogenous CCK released by the trypsin inhibitor diffuses through the lamina propria and activates the submucosal plexus; however, there is insufficient CCK to diffuse to the myenteric plexus. (Used after permission from Raboin *et al.*[288], appendix E).

Using an Ensure meal to cause the release of endogenous CCK resulted in a significant increase in c-Fos both in brainstem nuclei and in the SFO. Although, the increase in c-Fos in the brainstem was not blocked by prior administration of a CCK_1 or a CCK_2 antagonists (as previously shown [112]), c-Fos immunoreactivity in the SFO was partially blocked by pretreatment with a CCK_2 receptor antagonist. This suggests a role of CCK in meal induced c-Fos activation in the SFO. The difference between the effect of SBTI and the meal might be explained based on three possibilities. The first is that a meal may activate more than one co-factor to release endogenous CCK from intestinal I cells possibly leading to higher concentration of circulating CCK. The other possibility is that a meal causes a release of central CCK [293, 294] which might cause activation of the SFO by an indirect pathway through afferents connections to the SFO from other brain regions where central CCK might be released. A third possibility is that Ensure releases other gut peptides whose effects are additive with CCK to activate the SFO.

Consistent with the previous results in Chapter 2 using i.p. CCK and showing a CCK_2 dependent activation of the SFO, it was found that meal induced activation of the SFO is partially blocked by the CCK_2 receptor antagonist (L-365.260). Interestingly, the blockade was not complete as seen using i.p. CCK which suggests the contribution of other circulating hormones following feeding to the observed SFO activation. Examples of other satiety factors that may contribute to the observed residual c-Fos expression are nesfatin-1 [148] PYY [151] and amylin [159] which have been shown to activate SFO neurons.

In conclusion, using vagotomized rats, it was shown that CCK induced SFO activation does not require an intact vagal pathway which is different than the effect of CCK on the brainstem nuclei, AP and NTS. It is also shown that activation of the SFO following a meal is partially mediated by a CCK_2 receptor pathway; however, this effect was not observed by the induction of local CCK release using soy bean trypsin inhibitor.

Chapter 4 Effect of high fat diet on the activity of CVOs and their responsiveness to CCK and an angiotensin II receptor antagonist

4.1 Introduction

The results of the previous chapters show evidence of the responsiveness of the sensory CVOs; AP and SFO, to systemic CCK administration as well as meal-induced release of CCK. In order to test the general hypothesis in Chapter 1, the next step is to study the change in the activity of these CVOs in response to CCK in a high fat diet-induced model of obesity. The effect of the diet itself will also be explored using a shorter time of exposure to the high fat diet.

4.1.1 High fat diet induced obesity.

Obesity is defined as an increase in body fat as a result of a positive energy balance caused by genetic factors combined with environmental factors such as increased caloric intake and a sedentary lifestyle [319]. Obesity is a global epidemic, and is especially prevalent in Western countries. It represents a serious health problem leading to diabetes [14, 15], cardiovascular diseases, stroke [16, 17] and cancer [18]. The WHO defined obesity in 1998 to be a chronic disease due to its high risk of complications [13]. Since then, obesity has become a rich research area in order to investigate the pathophysiology and find possible treatments of this disease.

Several rodent models are used for the induction of obesity and to study its associated complications. Genetic animal models have specific mutations that lead to generation of a phenotype which spontaneously develop obesity. Examples of the genetic models are ob/ob and db/db mice, which were mutant strains discovered in

1950 and 1965, respectively [320]. They are characterized by an obesity phenotype and were used as a model for diabetes. It was not until the 1990s, after discovery of leptin and the cloning of the leptin receptor, that these mutants were characterized to have mutations in genes responsible for expression of leptin protein (ob/ob) or leptin receptor (db/db) [321, 322]. Treatment of ob/ob mice with leptin reverses its phenotype and results in normalized body weight [323]. In humans, many leptin deficiency syndromes are responsive to leptin replacement therapy, however, leptin mutations are not common in obese patients [320]. As most obese humans, who have normal genes for leptin and its receptor have been found to have elevated leptin levels [324] suggesting a form of leptin resistance [325], this model does not reflect the common pathophysiology of obesity.

In addition to genetic mutations, there are other models that utilize pharmacological intervention to induce obesity, such as injection of gold-thioglucose which induces a lesion in the ventromedial hypothalamus leading to hyperphagia and obesity [326], however, these models focus only on specific loci to study obesity and similarly do not replicate common forms of human obesity.

A major factor in the initiation of obesity is the type of the ingested food. Food rich in fat (≥ 30% fat of total energy) has been thought to be a leading cause of obesity [327, 328]. Studies have shown that high fat intake not only induces obesity in humans, but also in rats [329, 330] and mice [331, 332]. However, it is important to note that, intake of high fat diet alone may not be sufficient to induce obesity as genetic factors are important determinants of the tendency to store energy in the form of fat as a result

of high fat diet (HFD) [333, 334]. The first instance of induction of obesity using a HFD in rats was in 1949, when Ingle observed that rats fed a highly palatable semi-liquid diet became obese [335]. Since then, HFD-fed animals have been extensively used as experimental animal model of dietary obesity [336, 337]. Varying amounts of fat are used for the preparation of the HFD, ranging from 30 to 80% of total energy intake [338]. The HFD is prepared either by adding fat to the normal animal diet or by using fat and sugar rich supermarket food (cafeteria type diet). Several mechanisms were proposed to explain the effect of the HFD on body weight. Some of them are related to the nature of fat as a dietary component and some related to the specific reaction of the body to dietary fat [339]. The fat content of food has a smaller thermogenic efficiency (defined as the total energy required to digest, absorb and store a certain diet component) than the other components of a diet; which is 2-3% for fat, 6-10% for carbohydrates and 25-30% for proteins [339]. Consequently, fats have the greatest food efficiency (which is defined as weight gain (g) in response to 1 kilogram of food). Another factor related to the physical properties of fat is that fats cause less gastric distension compared to carbohydrates, for example, leading to a lower satiating effect [340]. In addition to these physiological effects associated with HFD, availability of fatty diet increases food intake due to its palatability in terms of texture and odor [341-343].

HFD has been shown to modulate the effect of circulating factors affecting energy balance and body weight. Leptin [344-346], ghrelin[347, 348] and insulin [349-351] are just a few examples of hormones found to be affected by consumption of diet rich in fat and its associated obesity. Alterations of the actions of CCK have also been

reported in HFD-induced obesity. For example, rats consuming the HFD have been shown to have modified physiological actions of CCK, such as reduction of the effect of i.p. CCK on gastric emptying [170], brainstem neuronal activation [163], and food intake [171]. Therefore, understanding any modified responsiveness of the sensory CVOs, which have important roles in energy balance regulation (as discussed in Chapter 1), in diet-induced obese rats is critical for understanding the potential roles of the CVOs in the development of obesity.

4.1.2 Sensory CVOs, high fat diet and obesity.

The responsiveness of the AP and the SFO to HFD or HFD-induced obesity has not been extensively studied. c-Fos immunoreactivity in the AP in response to CCK was attenuated in rats maintained on high fat diet for 2 weeks [352]. In another study, the same effect was observed in diet-induced obese rats fed high fat diet for 12 weeks [353]. Similarly, c-fos mRNA in the AP induced by oleoylethanolamide (an endogenous lipid compound that induces satiety after i.p. administration [354]) was attenuated by feeding the rats a HFD for 2 weeks [355]. In contrast to CCK, it was found that oxytocin (a neuropeptide involved in the control of food intake and autonomic function [356])-induced increase in c-Fos in the AP was enhanced in animals fed HFD [357].

Regarding the SFO, literature studying the effect of HFD on its activity is very limited. To my knowledge, only one study demonstrated the effect of HFD-induced obesity on the activity of microglia within the SFO [358]. They found that HFD increased microglial activation, which was accompanied by increased expression of the angiotensin receptor subtype 1. Another study by Smith and Ferguson investigated the

effect of direct injection of leptin into the SFO in lean versus diet-induced obese rats [153]. They found that leptin-induced reduction of blood pressure in lean rats was absent in obese rats. Therefore, the modified responsiveness of the sensory CVOs in diet-induced obese rats needs further study and is critical for understanding the potential roles of the CVOs in the development of obesity.

4.1.3 Role of renin angiotensin system in obesity.

The renin–angiotensin system (RAS) is an endocrine system which plays an important role in cardiovascular and renal physiology. When the RAS is overactivated, it leads to the induction and progression of cardiovascular diseases such as hypertension, atherosclerosis, heart failure, ischemic heart disease as well as renovascular disorders [359-361]. Renin and its precursor prorenin are both initiators of the RAS signaling cascade. Renin acts by converting angiotensinogen to angiotensin I (AngI), which is converted into Ang II by the action of the enzyme angiotensin converting enzyme (ACE) [362]. AngII is considered as the principal component of the RAS cascade. It has diverse physiological actions acting on the heart, kidney, blood vessels and the CNS in order to regulate blood pressure and fluid balance [363]. AngII acts on two subtypes of angiotensin receptors: angiotensin receptor subtype 1 (AT1R) and 2 (AT2R). These receptors belong to the G protein-coupled receptor superfamily. Two isoforms of the AT1 receptor (AT1a and AT1b) have been identified in rat and mouse and are pharmacologically indistinguishable. Both isoforms are selectively antagonized by losartan. AT1Rs in human, rat and mouse are formed of a 359 amino acid-polypeptide. The rat AT1 receptor is about 95% identical to the human AT1 receptor [362].

Recently, the RAS has been found to be implicated in body weight and energy balance regulation [364]. Components of the RAS were found to be expressed in adipose tissue [364] and their serum levels were reported to be elevated in obesity [365]. Pharmacological [358, 366] or genetic [367] manipulation of the RAS has been shown to affect body weight and energy expenditure. As renin and angiotensin peptides do not cross the blood brain barrier [368], sensory CVOs represents potential targets for their actions. Moreover, brain tissues, especially the sensory CVOs SFO and OVLT, express both of the angiotensin receptors and contain AngII [369]. Given the potential role of the SFO in the function of energy balance regulation (discussed in Chapter 1), the SFO might be a target for the RAS induced effects on body weight and energy balance.

4.1.4 Hypothesis and Aims.

In this Chapter, I examine the effect of HFD- induced obesity on the response of the AP and the SFO to exogenous CCK-8 using c-Fos immunoreactivity as a marker of neuronal activation in order to test the hypothesis that HFD induces changes within sensory CVOs that affect their responsiveness to CCK.

The aims of this chapter are to:

1. Study the effect of HFD feeding (5 weeks) as a model of obesity on the response of the AP and SFO to CCK.

The five week period was selected based on the fact that rats on high fat diet typically start to show an obese phenotype after 4 weeks [338]. However, this depends on the age of rats and percentage of fat used [370, 371]. In my study, I continuously monitored body weight change and found that after 2 weeks a significant increase of body weight

in HFD fed rats was observed compared to chow-fed controls. By week 4, the HFD group could also be distinguished into diet resistant (DR) and diet-induced obese (DIO) subgroups [372, 373] and hence I selected this time to study the effects of CCK.

In order to test the effect of the high fat content of the diet before induction of obesity and the initiation of metabolic changes, another study was carried examining the effect of a shorter term high fat feeding (2 weeks), thus the second aim was:

2. Study the effect of short term HFD feeding (2 weeks) on neural activity in the AP and the SFO.

The results of these two studies showed that the SFO had basal c-Fos immunoreactivity and because there is an evidence of increased RAS activity in response to high fat feeding, the third aim of the Chapter was to:

3. Study the effect of HFD feeding (2 weeks) combined with treatment with an angiotensin receptor 1 antagonist (losartan) on the c-Fos activity in the SFO.

4.2 Materials and Methods.

4.2.1 Animals.

Male Sprague-Dawley rats (120-150 gm) were purchased from Charles River (Montreal, Quebec, Canada). Upon arrival, they were housed under specific pathogen free conditions on a reversed 12-hour light-dark cycle with free access to food and water for one week. On the first day of feeding, rats were divided into two groups; one group was allowed standard laboratory chow (Laboratory rodent diet 5001, 13.5% kcal from fat, 58% kcal from carbohydrate and 28.5% kcal from protein; Lab Diet, St. Louis, MO, USA) and the other group allowed free access to a HFD (D12451, 45% kcal from fat, 35% kcal from carbohydrate and 20% kcal from protein; Research Diets Inc., New Brunswick, NJ, USA).

4.2.2 Compounds.

Cholecystokinin (sulphated CCK-8; catalog no. 069-03) was purchased from Phoenix® (Phoenix Pharmaceuticals, Burlingame, CA, USA). Losartan (Losartan potassium, Cat. No. 3798) was purchased from Tocris Bioscience.

4.2.3. HFD-induced obesity study.

A total of 34 age-matched rats were used in this experiment. On day 0, all rats were weighed and randomly assigned to two groups: chow (n=8, 210.6 gm ± 2.8) and high fat diet (HFD)-fed (n=26, 210.0gm ± 1.2). The mean starting body weight of the chow-fed group was not significantly different from that of the HFD group as shown in Figure 4.1. Body weight and food intake were continuously measured throughout the period of the study. To show the actual difference in energy intake, food intake was

calculated as caloric intake. Conversion was based on manufacturers' information so that 1 gm of chow = 3.36 Kcal and 1 gm of HFD = 4.73 Kcal.

Four weeks after initiation of HFD, body weights of all rats were arranged in a descending order and the top 10 (about 40%) rats according to their weight were assigned as DIO (546.6 gm ± 8.3) and those having the ten lowest body weights were assigned as DR (475.5 gm ± 7.9), according to methods used previously [372, 373]. Rats with body weights in-between the DR and the DIO were excluded from the study (n=6). Retrospective calculation of food intake of DIO and DR groups was done to calculate the difference in food intake since the start of the study.

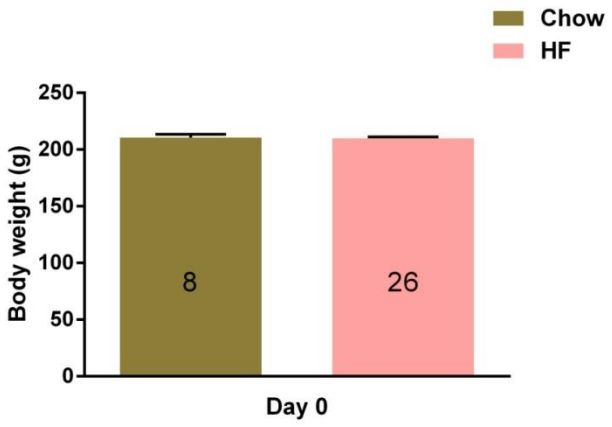


***Figure 4.1*: Initial body *weights* of chow-fed and HFD-fed groups**.

Bar graphs comparing mean body weight of the chow-fed and HFD-fed groups at the beginning of the 5 weeks HFD feeding study. Data are presented as the mean ± S.E.M. There is no significant difference between the groups.

The experimental design of this study is outlined in Figure 4.2 showing the timeline of the study.

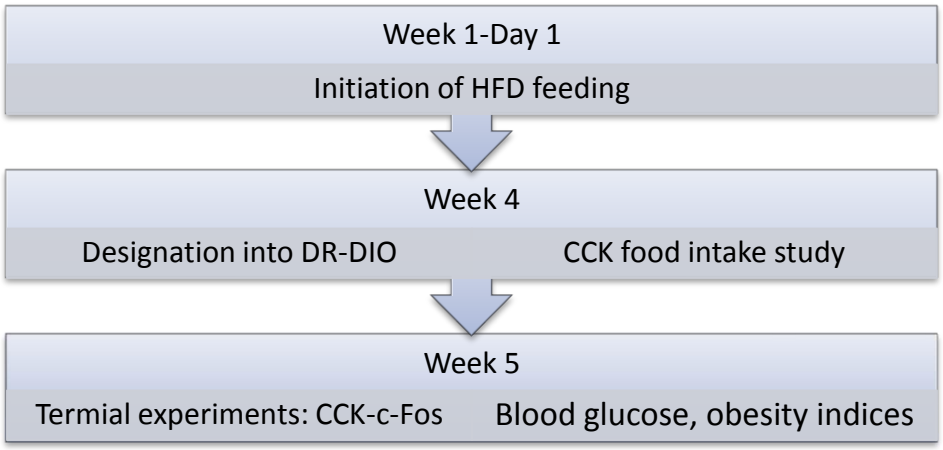


Figure 4.2: A diagram summarizing timeline of HFD-induced obesity study.

4.2.3.1 Effect of i.p.CCK-8 on food intake.

At week 4 and three days before the terminal experiment, i.p. CCK-8 (16 μg/kg) was tested for its ability to reduce food intake in both chow and HFD fed rats by following the same procedures outlined in Chapter 2.

4.2.3.2 Measurement of fasting blood glucose and obesity indices.

Prior to the terminal CCK experiment, rats were fasted overnight with free access to water. On the day of experiment, rats from each group (chow, DR and DIO) were divided into two groups; one receiving i.p vehicle and the other receiving CCK-8 (16 μg/kg, i.p.). Ninety minutes after injection, the following parameters were measured:

1- Body weight and naso-anal body length ratio as an obesity index [374].
2- Fasting blood glucose using a commercially available meter (One touch Ultra glucometer (LifeScan, Mountain View, CA) [375-377].

3- Epididymal fat pad and gastrocnemius muscle were dissected and weighed from each rat to use their ratio as an adiposity index [378, 379].

4.2.3.3 Effect of i.p.CCK-8 on c-Fos immunoreactivity in the AP and in the SFO.

At the end of the 90 minutes after CCK injection, rats were perfused, the brains were dissected and fixed overnight in 4% PFA. The procedure for c-Fos immunohistochemistry was then followed as previously outlined in Chapter 2.

4.2.4 Two week-HFD feeding study.

To test the effect of dietary fat alone before induction of significant obesity, a shorter term HFD feeding study was performed. A total of 16 age matched rats were used in this study. At day 0, all rats were weighed and randomly assigned to two groups: chow (n=8, 248.8 gm ± 2.3) and HFD-fed (n=8, 244.1 gm ± 2.2). As shown in Figure 4.3, the mean initial body weight of chow-fed group was not significantly different compared to the mean body weight of the HFD-fed group. After two weeks of HFD feeding, the HFD-fed group showed a significantly higher body weight than rats on chow ($p < 0.05$; Figure 4.4). On the day of experimentation, overnight fasted rats were subjected to the same procedure described above in the 5 week HFD feeding study.

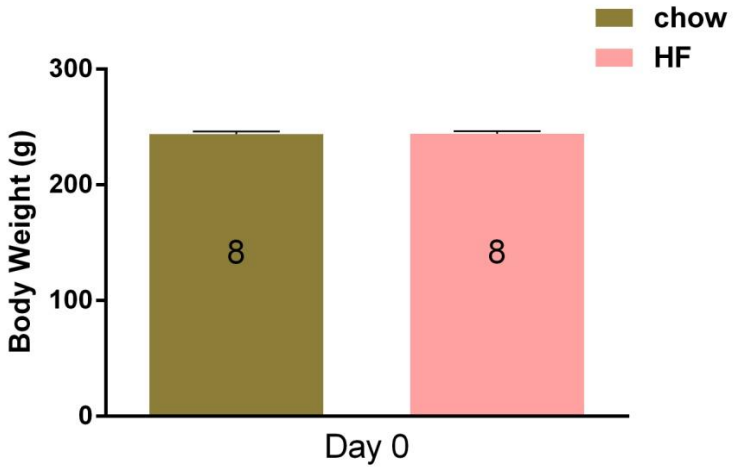


Figure 4.3: Initial body weights of chow-fed and HFD-fed groups (2 weeks study).

Bar graphs showing mean body weight between the chow-fed and HFD-fed groups at the beginning of the 2 weeks HF feeding study. Data are presented as the mean ± S.E.M. There are no significant differences between the groups.

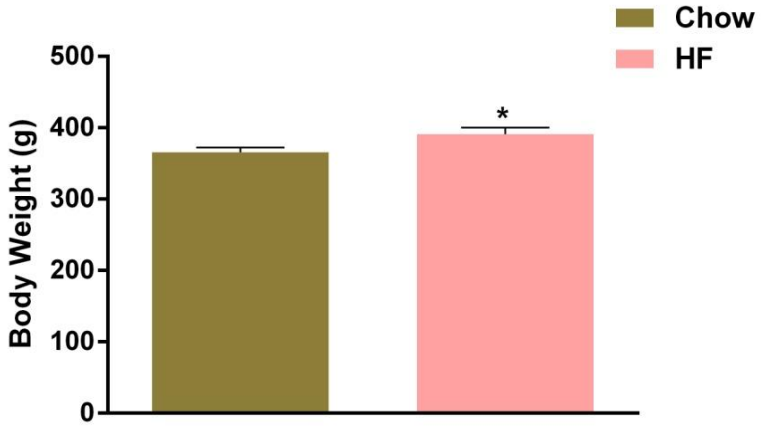


***Figure 4.4*: Body weight of chow-and HFD-fed rats after 2 weeks of HFD feeding.**

Data are presented as the mean ±S.E.M. *p< 0.05 represents significant difference from chow group.

4.2.5 Two week-HFD feeding with or without losartan study.

As stated in the introduction of this chapter, RAS activity is increased in response to HFD and since the results of the first two studies showed an increase in basal c-Fos expression in the SFO in HFD groups, I wanted to study if blocking AT1R using losartan will result in a reduction of this effect.

In order to test the effect of losartan treatment on two weeks HFD feeding, a total of 20 age-matched rats were used in the study. Basal daily water intake was measured for one week before the beginning of feeding and losartan treatment. At day 0 (Figure 4.5), all rats were weighed and randomly assigned to four groups: chow-water (n=5, 252.2 gm ± 5.2), chow-losartan (n=5, 250.0 gm ± 5.1), HFD-water (n=5, 250.2 gm ± 4.8) and HFD-losartan (n=5, 252.6 gm ± 7.0). Rats assigned to the chow-losartan and the HFD-losartan groups received losartan in drinking water in a dose of 30 mg/kg/day. The dose of losartan was based on previous literature [380-383] and it was shown to block AngII induced cardiovascular and metabolic effects both peripherally and centrally. Water and food intakes as well as body weight were continuously measured for all groups. After two weeks, overnight fasted rats were subjected to the same procedure described above.

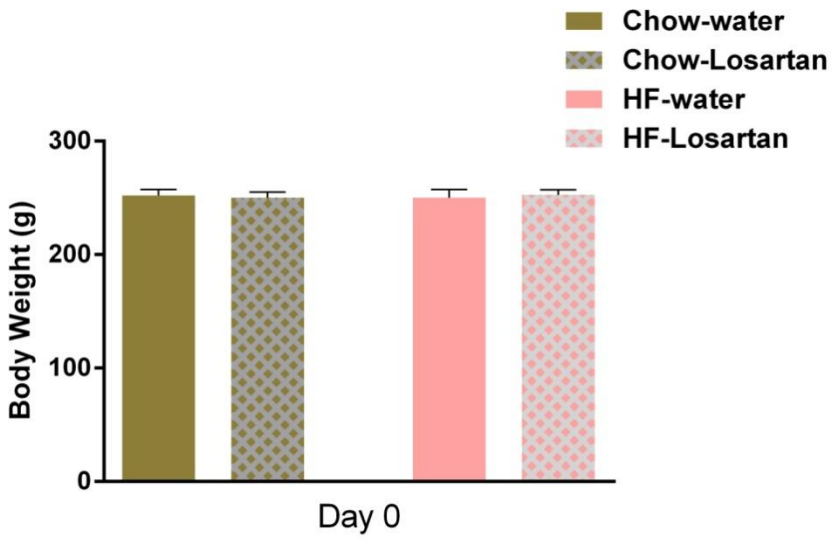


***Figure 4.5*: Initial body weights of chow-fed and HFD-fed groups**.

Bar graphs showing mean body weight between the four groups at the beginning of the 2 weeks HF feeding study. Data are presented as the mean ± S.E.M. There were no significant differences between the groups.

4.2.6 Iba-1 immunohistochemistry.

As a result of the high basal c-Fos immunoreactivity observed in the high fat diet fed groups, I wanted to ask if this basal c-Fos expression is mediated by increased activation of the microglia. Activated microglia have been shown previously in response to high fat diet [384]. Accordingly, some floating sections containing SFO were used for staining of microglia using an antibody recognizing ionized calcium binding adaptor molecule 1 (Iba-1, 1:1000; Wako Pure Chemical Industries, Osaka, Japan) as described before [384-386].

4.2.7 Statistics.

All statistical analyses were performed using GraphPad Prism (version 6.0; San Diego, CA, USA). When comparing two groups, data were analyzed using Student's t test. Analysis of Variance (ANOVA) test was used for multiple comparisons followed by Bonferroni post tests. In the experiment where losartan was used, body weight, food and water intake were analyzed by two-way ANOVA with time as a repeated measure and treatment as a between-group factor.

4.3 Results

4.3.1 HFD-induced obesity study.

4.3.1.1 Body weight and food intake of chow and HFD-fed groups.

As shown in Figure 4.6A, food intake (expressed as g/100 g body weight) shows that HFD-fed rats ate a smaller number of grams of food, however, when converting g to Kcal as shown in (B), it is obvious that HFD-fed rats had a greater caloric intake than chow-fed controls. After two weeks of HFD feeding the HFD group started to show a significantly higher body weight compared to the chow-fed rats (369.6 ± 6.3 g for chow versus 399.8 ± 4.8 g for HFD-fed rats). The difference in body weight continued to show significant differences ($p < 0.001$) throughout the study as shown in Figure 4.7.

Four weeks after the initiation HFD feeding, the rats were designated as being diet-induced obese (DIO) or diet-resistant (DR) based on their body weight [372, 373] as described in the methods section. As shown in Figure 4.8 , the mean body weight of the DR and the DIO groups was significantly different from the chow-fed group ($p < 0.05$ and $p < 0.001$; respectively) and each other. Retrospective calculation of food intake of DIO and DR groups revealed that DR rats consumed less during the 4 weeks feeding period (Figure 4.9).

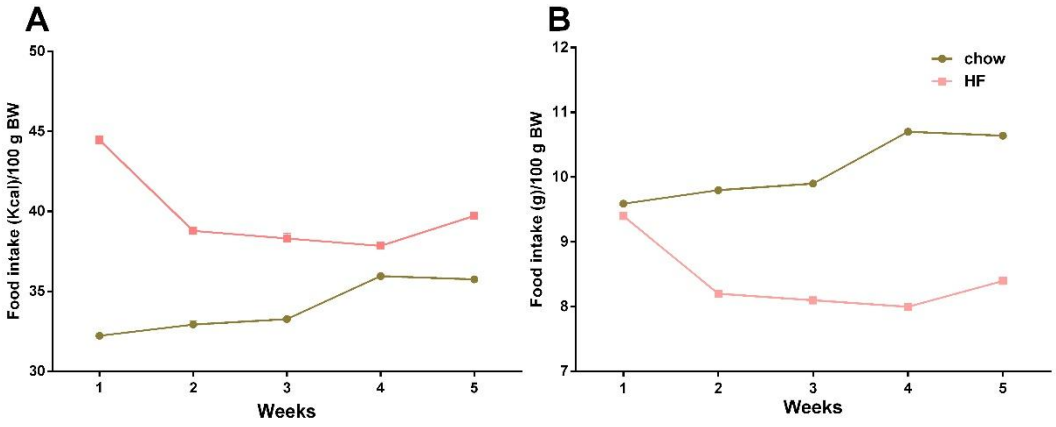


***Figure 4.6*: Weekly food intake for chow and HFD-fed rats presented as Kcal/ 100gm body weight; (A) and g/100 gm body weight (B).**

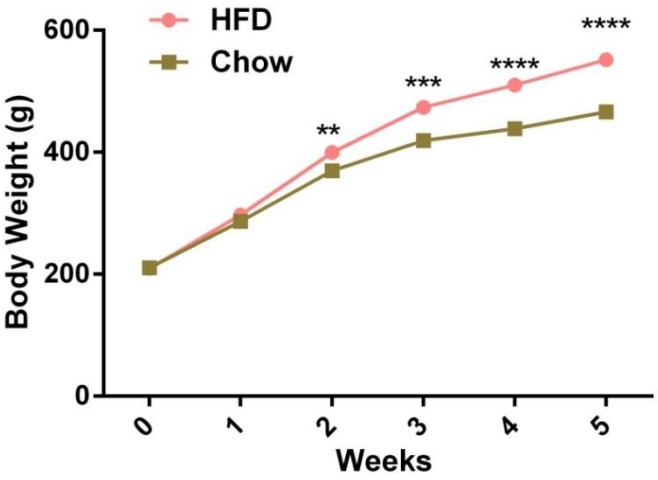


***Figure 4.7*: Weekly body weight of chow-fed and HFD-fed rats.**

Data are presented as the mean ± S.E.M, **p<0.01 and *** p<0.001 represent a significant difference from the chow-fed group.

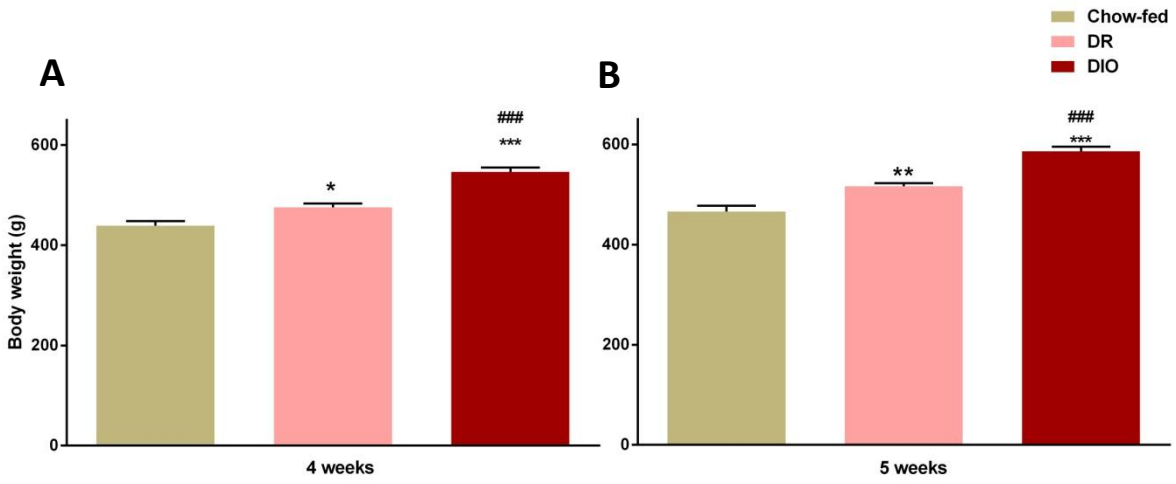


***Figure 4.8*: Body weight of chow-fed, DR and DIO rats after 4 and 5 weeks of HFD feeding.**

Data are presented as the mean ± S.E.M, *p<0.05, **p<0.01 and *** p<0.001 represent significant difference from the chow-fed group. ### p< 0.001 represents a significant difference from the DR group.

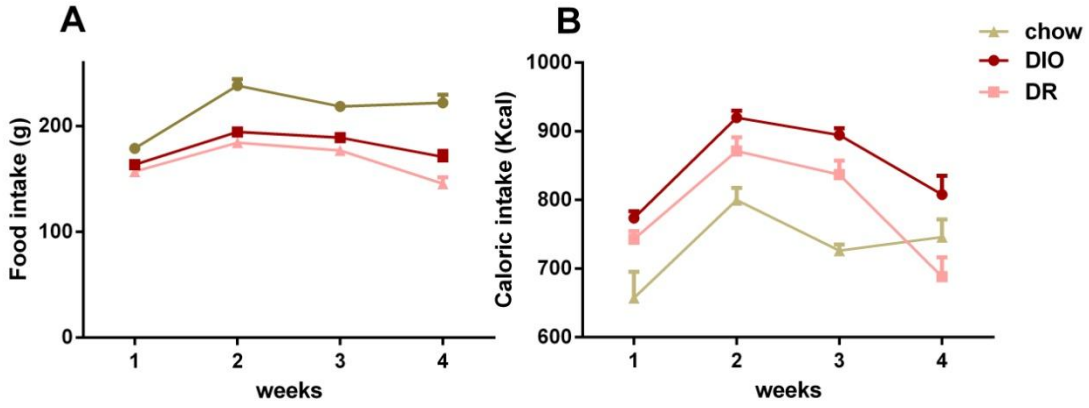


***Figure 4.9:* Retrospective calculation of weekly food intake for chow, DR and DIO rats expressed as weight (g; A) and caloric intake (Kcal; B).**

4.3.1.2 Fasting blood glucose and obesity indices of chow and HF fed animals.

As shown in Figure 4.10, DIO rats had a significantly higher fasting blood glucose compared to chow-fed or DR rats. The blood glucose of DIO group was 8.2 mM ± 0.6 compared to 6.4 ± 0.5 and 6.6 ± 0.3 for chow and DR groups, respectively. For reference, normal blood glucose levels range from 5.5-6.5 mM, while rats are considered to be diabetic with a blood glucose level > 9.5 mM [387]. Thus, DIO rats in this study are showing mild hyperglycemia but are not diabetic. Two obesity indices were used as a measure of increased body weight in response to HFD feeding; body weight/naso-anal length ratio and the ratio of the weight of epididymal fat pad/gastrocnemius muscle. Chow-fed rats showed a significantly lower body weight/nasoanal length ratio compared to the DR group ($p < 0.05$) and DIO group ($p<0.001$), as shown in Figure 4.11. Figure 4.12 compares fat pad to muscle ratio between the three groups, showing a significantly higher ratio ($p<0.001$) in both DR and DIO groups compared to chow-fed group. Representative photos of epididymal fat pads and gastrocnemius muscles showing the difference between the fat pad and muscles among chow-fed, DR and DIO rats are shown in Figure 4.13.

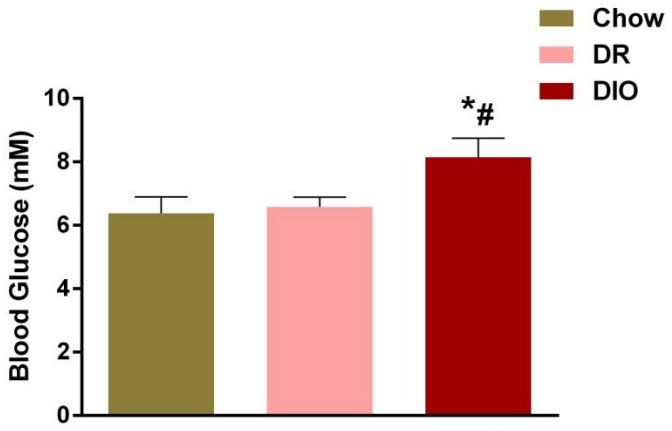


***Figure 4.10*: Fasting blood glucose of chow-fed, DR and DIO groups.**

Bar graphs presenting the fasting blood glucose of the chow-fed and HFD-fed groups (DR and DIO) after 5 weeks of high fat diet feeding. Data are presented as the mean ± S.E.M. *p<0.05 represents a significant difference from chow-fed group and #p<0.05 represents a significant difference from DR group.

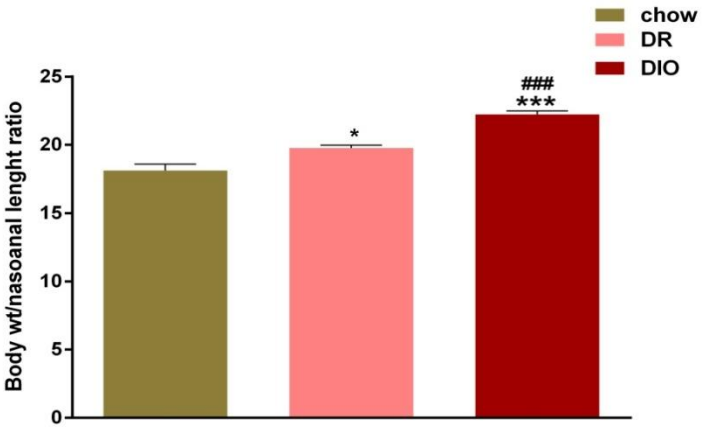


***Figure 4.11*: Ratio between body weight and naso-anal length in chow, DR and DIO groups.**

Data are presented as the mean ± S.E.M. *p<0.05 and ***p<0.001 represent significant difference from chow-fed group and ###p<0.001 represents a significant difference from DR group.

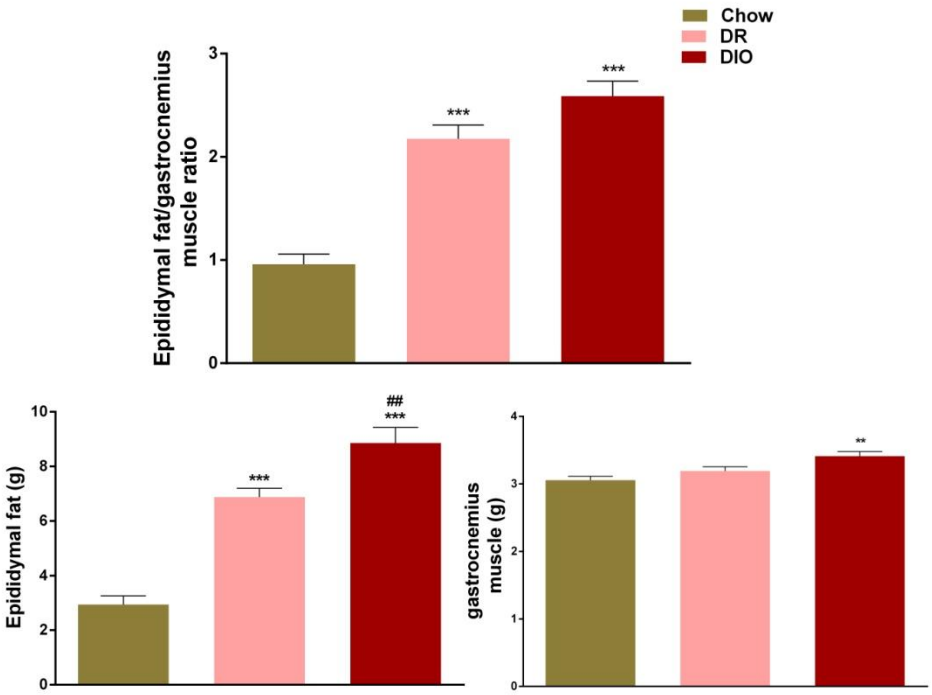


Figure 4.12: **Epididymal** ***fat to gastrocnemius muscle ratio in Chow, DR and DIO group after 5 weeks of HFD feeding.***

Bar graphs showing the difference in epididymal fat gastrocnemius muscle ratio in the chow-fed and HFD-fed (DR and DIO) groups (Upper panel) and individual comparisons of epididymal fat weight and gastrocnemius muscle weights between the three groups (lower panel).

Data are presented as the mean ± S.E.M. **p<0.01 and ***p<0.001 represent significant differences from the chow-fed group and ##p<0.01 represents a significant difference from the DR group.

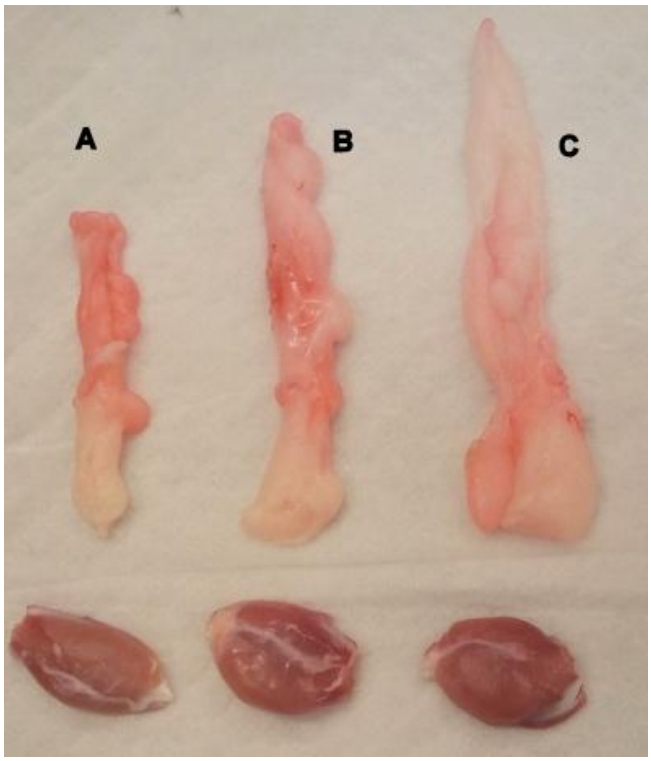


Figure 4.13: Comparison between the epididymal fat pads and gastrocnemius muscles of chow-fed, DR and DIO rats.

Representative showing epididymal fat pad (upper panel) and gastrocnemius muscle (lower panel) as dissected from a chow-fed (A), DR (B) and DIO rat (C).

4.3.1.3 Effect of CCK on food intake after 4 weeks of HFD.

As shown in Figure 4.14, intraperitoneal CCK-8 caused a significant reduction in 30 min caloric intake in chow fed animals. Treatment with CCK resulted in approximately 35% reduction in food intake in chow-fed rats; however, CCK failed to induce any significant effect in eitherHFD-fed groups, DR or DIO animals.

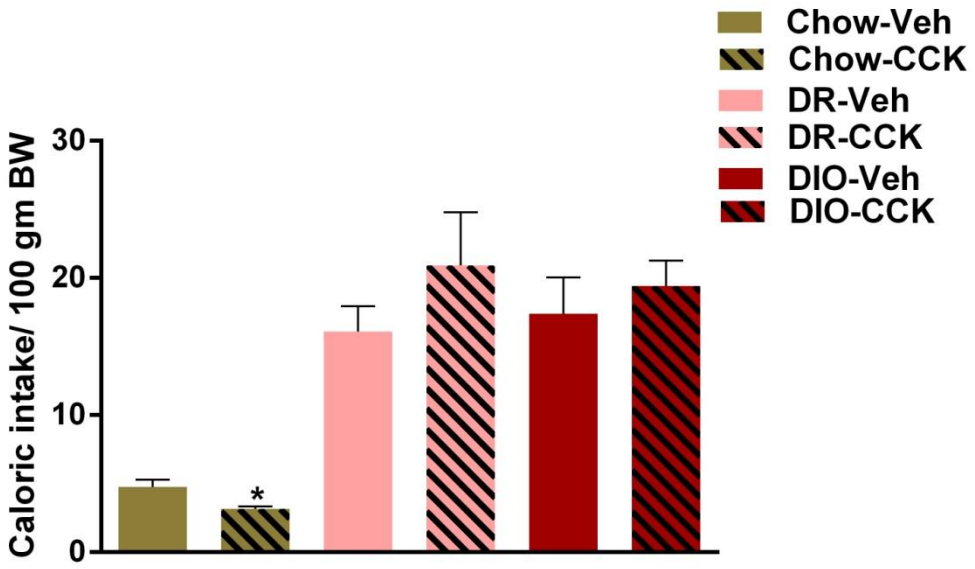


Figure 4.14: **Effect of CCK (16 μg/kg, i.p.) on food intake of chow-fed, DR and DIO rats.** Data are presented as the mean ±S.E.M. *p< 0.05 represents a significant difference from chow-vehicle control.

4.3.1.4 Effect of HFD-induced obesity on c-Fos immunoreactivity.

As shown in Figure 4.15 A, in chow-fed rats, CCK did not significantly increase c-Fos immunoreactivity in the AP, however, c-Fos in the NTS was significantly increased in response to CCK. Although the increase in c-Fos in the NTS was significant, the number of c-Fos immunoreactive neurons is obviously less than the number obtained using the same dose of CCK but in younger and lighter animals as previously shown in Chapter 2 (c-Fos count was > 250 in younger animals compared to a mean of 65 in older rats). It is important to note that the lack of significant response in c-Fos in the AP and the diminished response in the NTS are reflective to a reduced CCK inhibition of food intake which was only a 35% reduction in older animals compared to 70% in younger animals (Chapter 2). This observed age- and body weight-related change in sensitivity to CCK effect has been described previously by Balasko *et al.* in Wistar rats [388]. In HFD-fed rats (DR and DIO), there is a complete loss of significant c-Fos activation both in the AP and the NTS consistent with what is seen with the effect of CCK on food intake in both groups.

As shown in Figure 4.16 in the SFO, treatment with CCK induced a significant increase in the number of c-Fos immunoreactive neurons in the chow-fed rats (10.6 ± 2.6 versus 34.3 ± 4.8 for vehicle- and CCK- treated chow rats, respectively). However, in the DR and DIO rats, there was an increased c-Fos expression in vehicle treated groups which was not significantly different when compared to chow/CCK treated rats. This increased basal c-Fos made it difficult to comment on the observed non-significant increase seen with CCK treatment in these two groups and conclude that there was an

attenuated response to CCK in DR and DIO groups. As presented in Figure 4.16 B, the pattern of c-Fos expression in the SFO of DIO and DR was not specific to the core or the shell region of the SFO.

So the next step was to perform a shorter term HFD feeding (2 weeks) to test if this basal SFO activation will be seen with the shorter duration of HFD feeding.

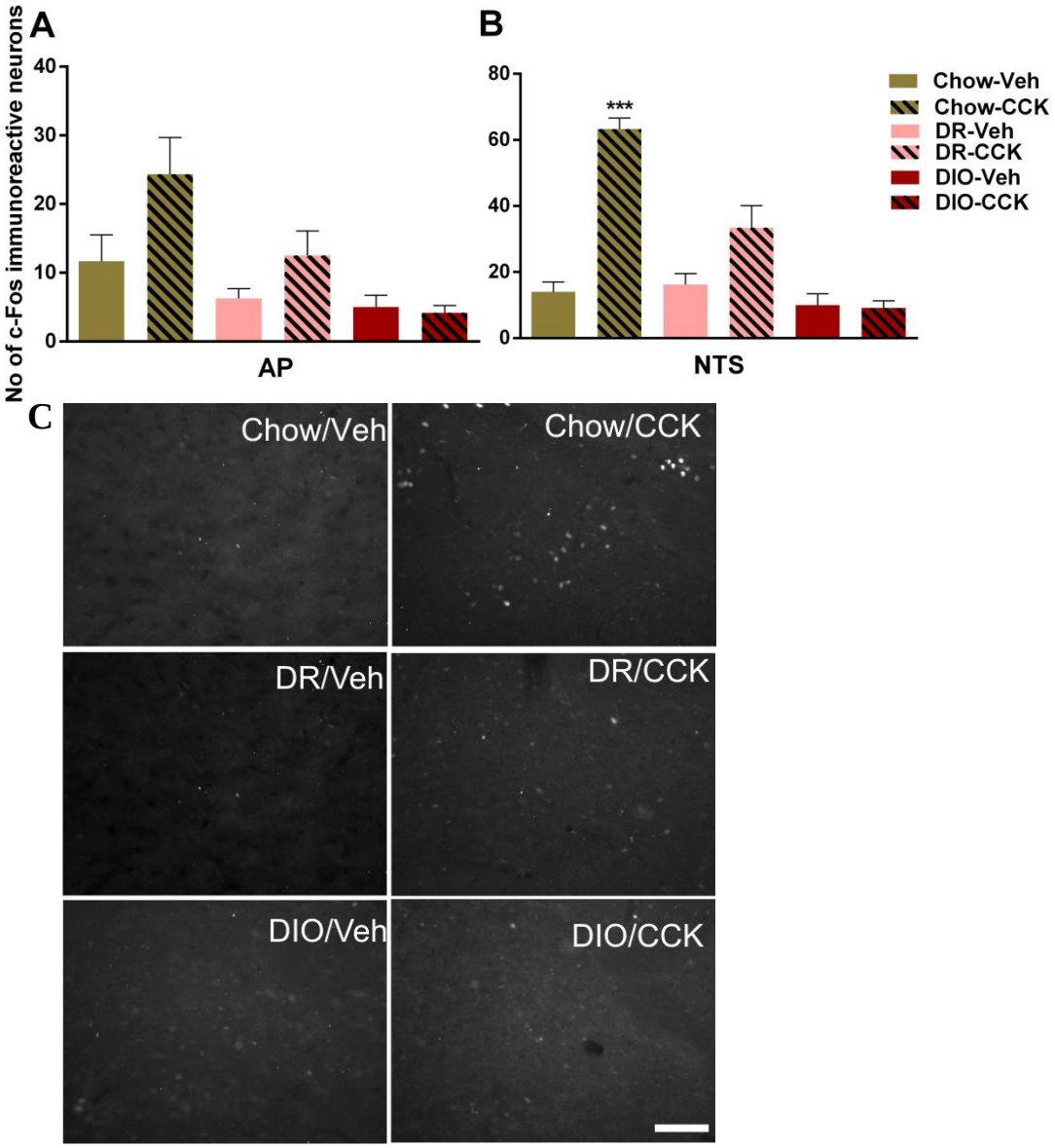


***Figure 4.15*: Effect of 5 weeks HFD on CCK-induced c-Fos expression in the AP and NTS.** Bar graphs comparing the number of c-Fos immunoreactive cells in the AP (A) and the NTS (B) in chow-fed, DR and DIO groups treated with vehicle (Chow/Veh, DR/Veh and DIO/Veh, respectively) or treated with CCK (16 µg/kg; Chow/CCK, DR/CCK and DIO/CCK), Data are presented as the mean ± S.E.M, *** $p<0.001$ represents a significant difference from the respective vehicle control. Representative immunofluorescence micrographs for all groups in the NTS are shown in C. Scale bar = 100 µm.

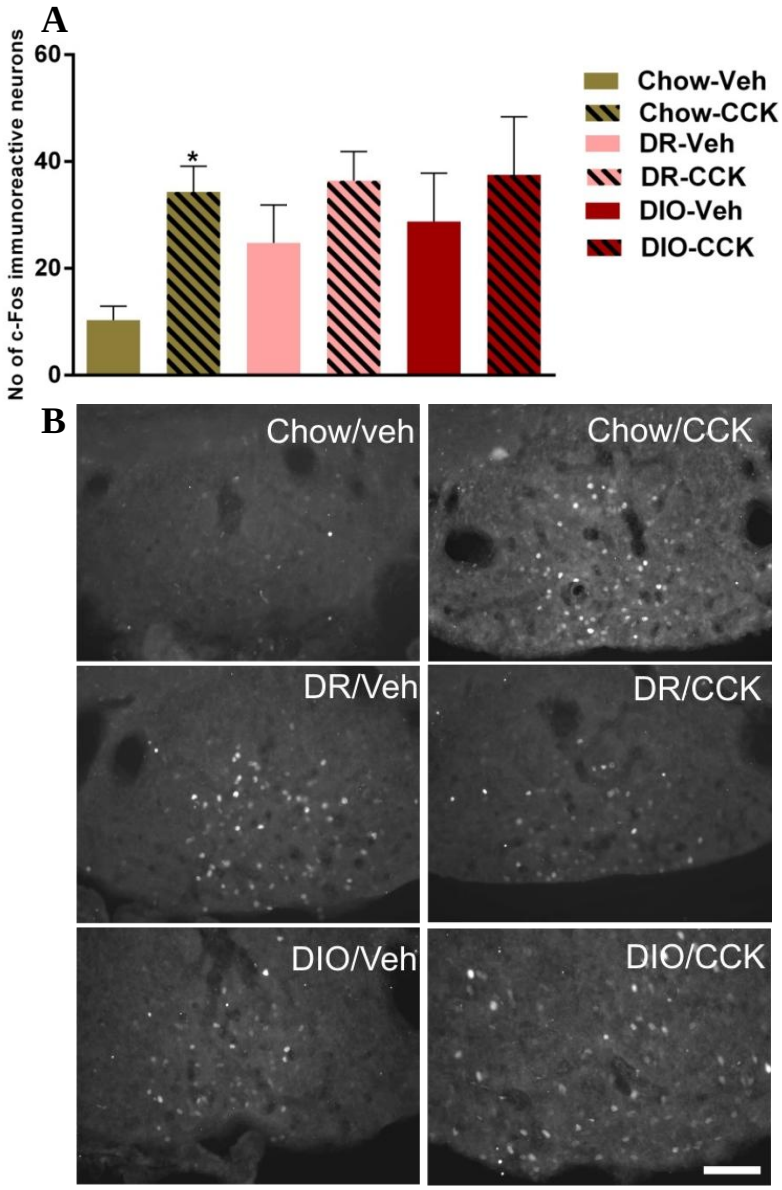


***Figure 4.16*: Effect of 5 weeks HFD on CCK-induced c-Fos expression in the SFO.**

Bar graphs comparing the number of c-Fos immunoreactive cells in the SFO (A) in chow-fed, DR and DIO groups treated with vehicle (Chow/Veh, DR/Veh and DIO/Veh, respectively) or treated with CCK (16 µg/kg; Chow/CCK, DR/CCK and DIO/CCK), Data are presented as the mean ± S.E.M, * $p<0.05$ represents a significant difference from the respective vehicle control. Representative immunofluorescence micrographs for all groups in the SFO are shown in B. Scale bar = 100 µm.

4.3.2 Two weeks high fat feeding study

4.3.2.1 Fasting blood glucose and obesity indices of chow and HF fed animals.

As shown in Figure 4.17, fasting blood glucose was not significantly different between chow and HFD rats. The blood glucose levels for chow and HFD rats were 7.1 ± 0.2 and 7.6 ± 0.3, respectively. Body weight/naso-anal length ratio and epididymal fat pad/gastrocnemius muscle ratio are presented in Figure 4.18 and Figure 4.19, respectively. After two weeks on a high fat diet, chow-fed rats showed a significantly lower body weight/naso-anal length ratio compared to HFD group ($p < 0.05$). Figure 4.19 compares fat pad to muscle ratio between the three groups, showing a significantly higher ratio ($p<0.001$) in the HFD group compared to the chow-fed group.

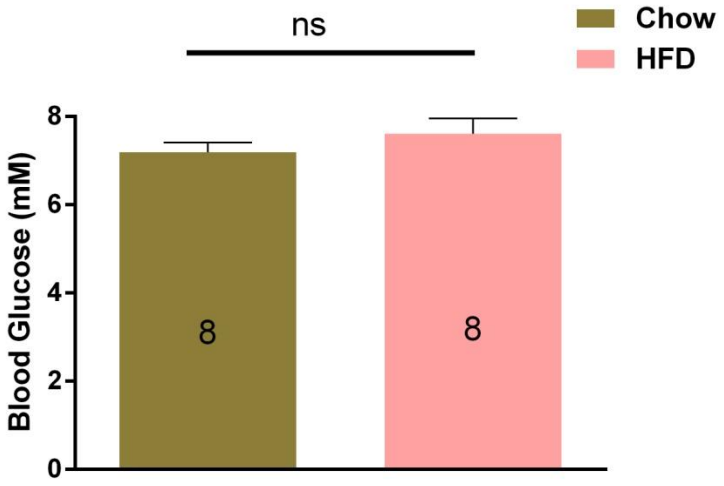


***Figure 4.17*: Fasting blood glucose of chow-and HFD-fed groups.**

Bar graphs presenting the fasting blood glucose of the chow-fed and HFD-fed groups after 2 weeks of high fat diet feeding. Data are presented as the mean ± S.E.M.

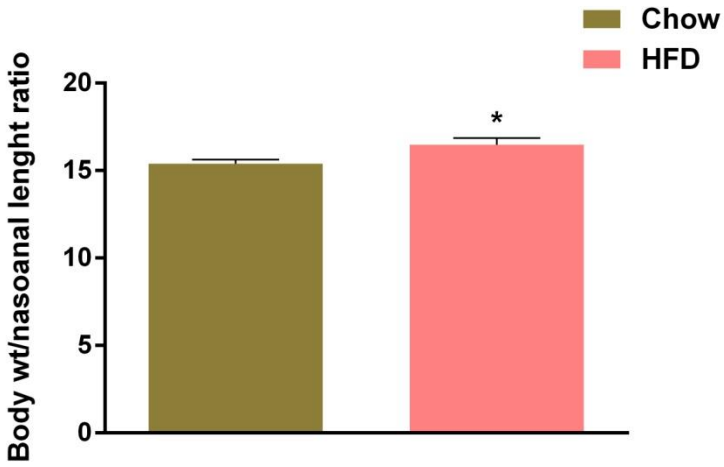


***Figure 4.18*: Ratio between body weight and naso-anal length in chow and HFD groups.**

Data are presented as the mean ± S.E.M. *$p<0.05$ represents a significant difference from chow-fed group.

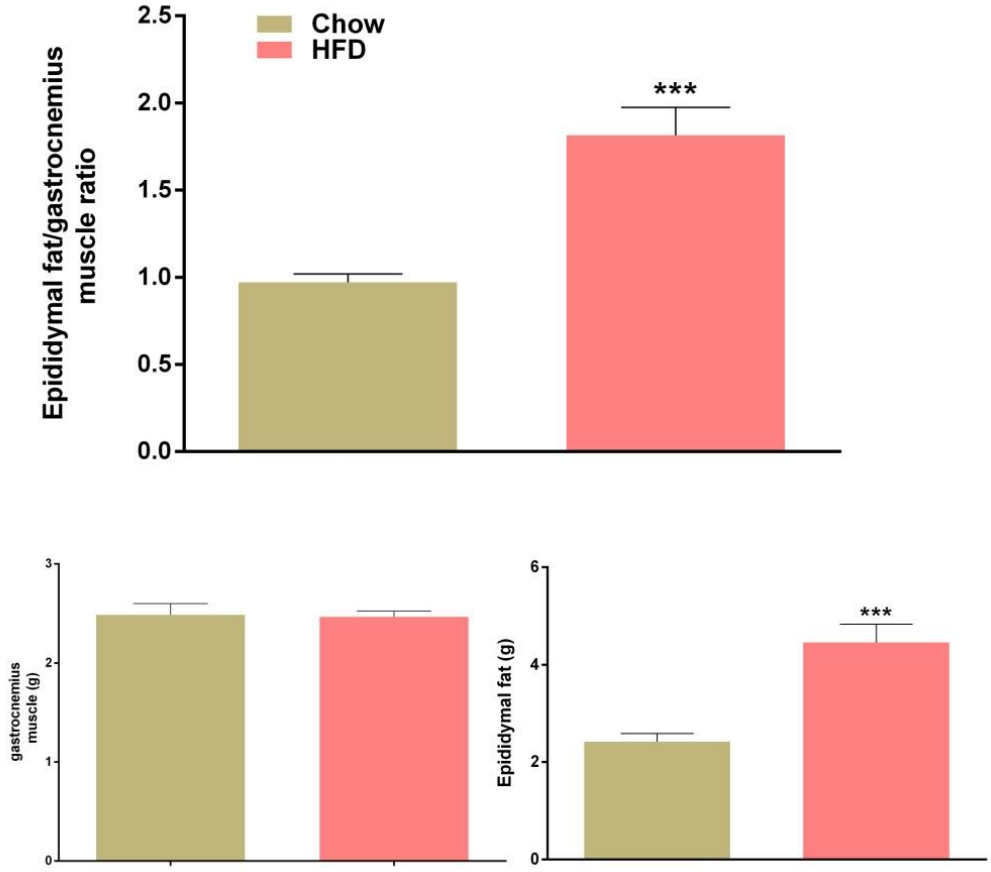


***Figure 4.19*: Epididymal fat gastrocnemius muscle ratio in Chow and HFD groups after 2 weeks of HFD feeding.**

Bar graphs showing the difference in epididymal fat gastrocnemius muscle ratio in the chow- and HFD-fed groups (Upper panel) and individual comparisons of epididymal fat weight and gastrocnemius muscle weights between the two groups (lower panel).

Data are presented as the mean ± S.E.M. ***$p<0.001$ represents significant difference from chow-fed group.

4.3.2.2 Effect of 2 weeks HFD on c-Fos immunoreactivity.

The effect of shorter term high fat diet feeding (2 weeks) on the c-Fos expression was studied in the AP (which did not show basal activation in response to the 5 weeks high fat diet feeding) and the SFO. As shown in Figure 4.20 A and B, there was no significant increase in basal c-Fos immunoreactivity in the AP in the HFD group compared to the chow-fed group. However, a significant increase in basal c-Fos expression was observed in the SFO of the HFD fed rats compared to chow-fed rats (10.5 ± 2.1 neurons versus 33.5 ± 3.6 for chow and HFD-fed groups, respectively).

4.3.3 Effect of HFD-induced obesity and 2 weeks HFD on microglial activation.

A recent study (2014) reported that HFD feeding in mice leads to microglial activation within the SFO and this activation might be due to the activated renin-angiotensin system [384]. Microglia have important functions as the central nervous system's resident immune cells which sense and respond to a wide variety of threats within the brain, including obesity. When activated, microglia undergoes morphological changes that allow for quantitative and qualitative assessment of their activity [389, 390].

Accordingly, I stained some of the SFO sections from both HFD feeding studies to examine if these results can be replicated in rats under our experimental conditions. As shown in Figure 4.21, microglial activation, evident as increased microglial size, was seen in the SFO of both short term and long term HFD fed rats, being more prominent in the 5 week-study. The observed microglial activation might be linked to the basal c-Fos observed in the SFO in high fat diet fed rats.

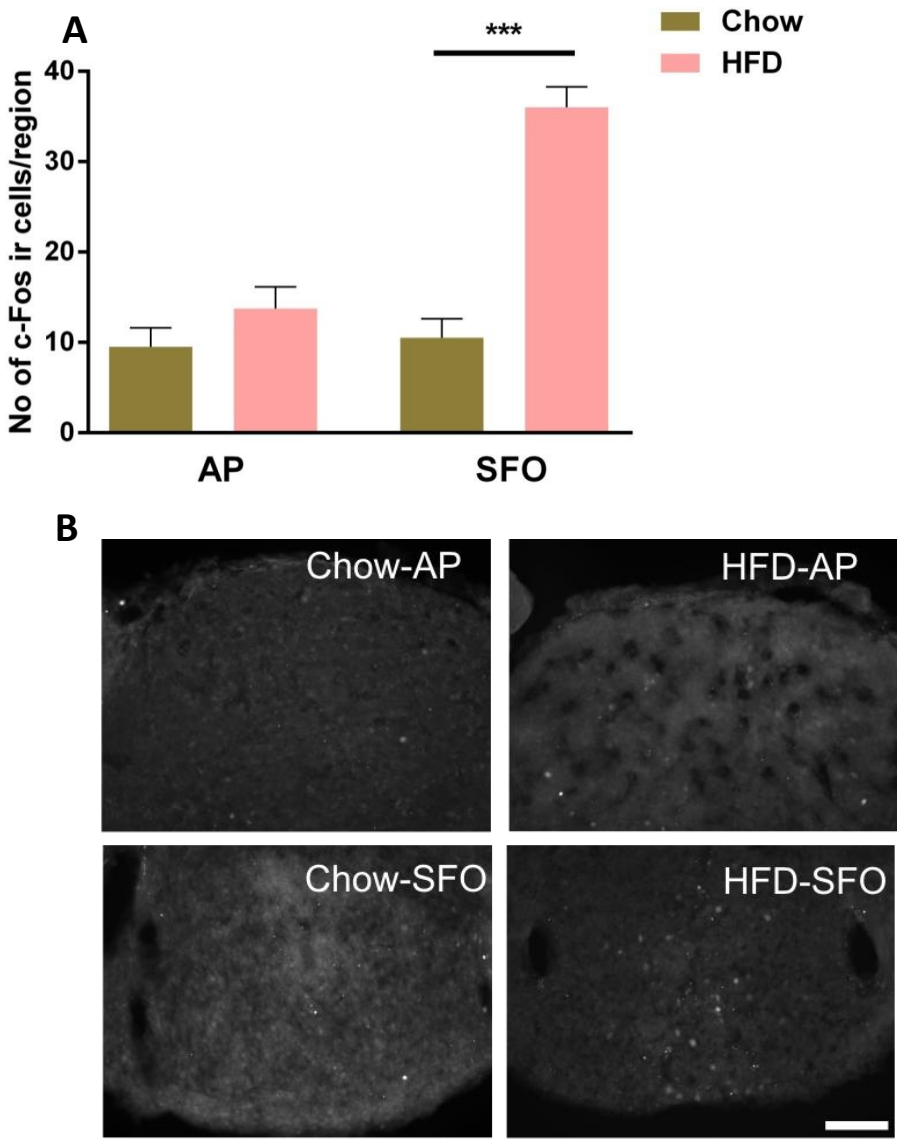


Figure 4.20: **Effect of 2 weeks HFD on basal c-Fos expression in the AP and SFO.**

Bar graphs comparing the number of c-Fos immunoreactive cells in the AP and the SFO in chow-fed and HFD groups (A). Data are presented as the mean ± S.E.M, *** $p<0.001$ represents a significant difference from the chow control.

Representative immunofluorescence micrographs for all groups in the AP (upper panel) and the SFO (lower panel) are shown in B. Scale bar = 100 µm.

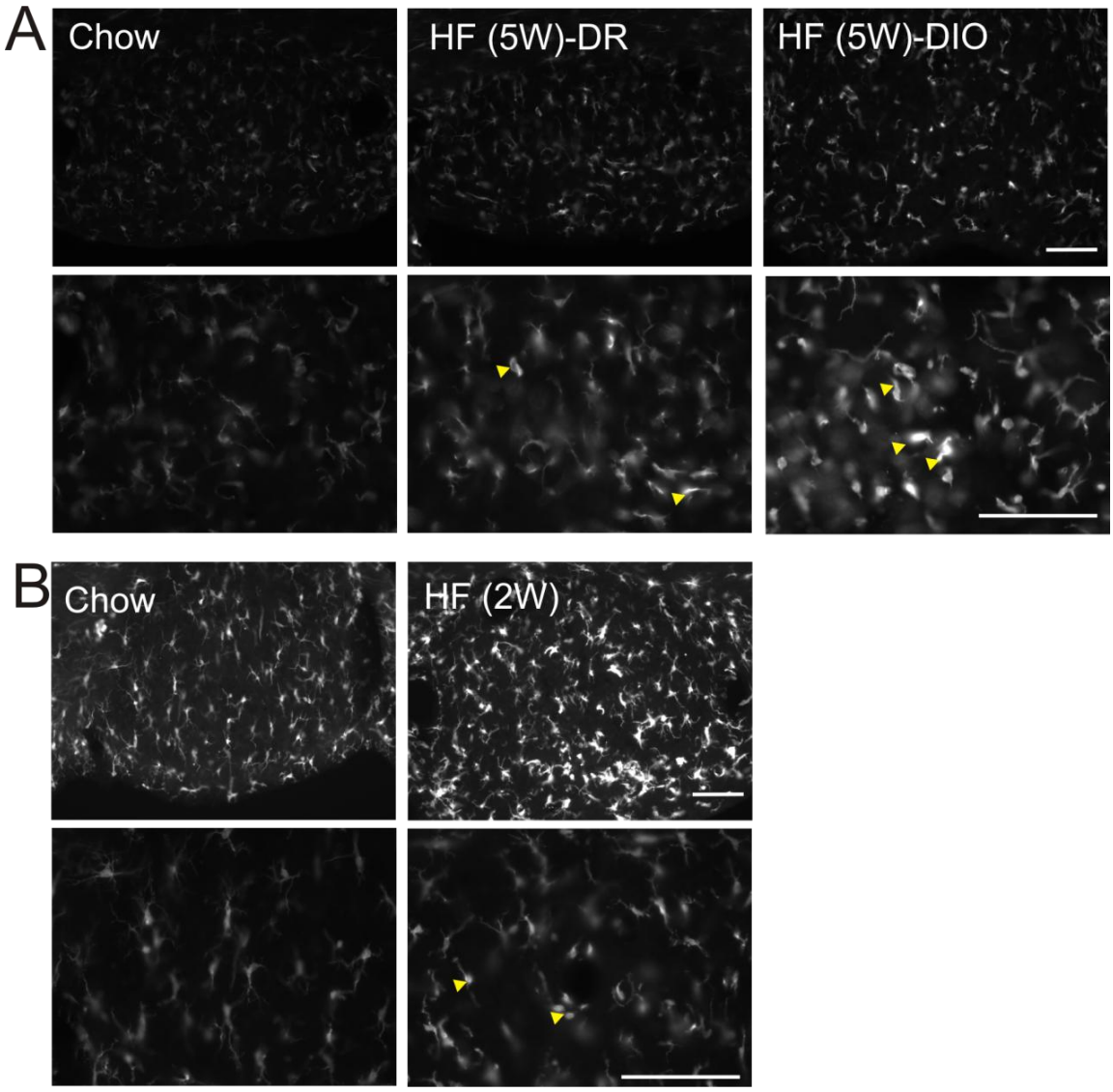


Figure 4.21: **Iba-1 immunoreactivity in the SFO of chow and HFD-fed rats after 5 weeks and 2 weeks on high fat diet.**

Representative photomicrographs showing Iba-1 immunoreactivity in the SFO of rats fed chow and HFD (5 weeks and 2 weeks, A and B, respectively). Higher magnification micrographs are shown in the lower panel with the yellow arrow heads pointing at some activated microglia. Scale bar = 100 µm.

4.3.4 Two weeks HFD feeding study with or without losartan treatment

As shown above in this Chapter, basal c-Fos expression was observed in the SFO in response to HFD feeding (5 weeks and 2 weeks) and according to a previous report [384], increased angiotensin activity within the SFO is observed in response to high fat diet. Accordingly, the next step was to study the effect of the non-peptide AT1R antagonist losartan given to the rats in their drinking water in a daily dose of 30 mg/kg for the 2 weeks on the HFD. The effects of HFD with and without losartan treatment on food intake, water intake, body weight and obesity indices as well as c-Fos expression will be presented in this section.

4.3.4.1 Food intake, water intake and body weight of chow and HFD fed rats with or without losartan treatment.

As shown in Figure 4.22 A, daily losartan treatment in drinking water did not affect average daily food intake in the chow-losartan group compared to the chow group receiving plain water. It also did not affect food intake between groups fed HFD with losartan in drinking water and those fed HFD and drinking plain water. In addition to food intake, treatment with losartan did not affect daily water intake for chow or HFD groups as shown in Figure 4.22 B. Noteworthy, both HFD fed groups showed a reduced daily water intake compared to the chow-fed groups.

Interestingly, although losartan did not affect food intake, rats on high fat diet with losartan in their drinking water showed a significantly lower body weight compared to HFD fed rats drinking plain water. This significant reduction was observed after 8 days on losartan treatment ($p < 0.01$) and continued to the end of the 2 week study, where

the mean body weight for the HFD-control group reached 383.6 gm ± 10.6 compared to 338.6 gm ± 7.8 for HFD-losartan group (Figure 4.23). On the other hand, losartan in the drinking water of chow-fed rats did not affect body weight at any time point. The body weight of both chow-fed groups only showed significant differences compared to HFD-control group towards the end of the two week study, reaching 356.6 ± 4.3, 358.5 ± 10.1 and 383.6 ± 10.6 g for chow-control, chow-losartan and HFD-control, respectively.

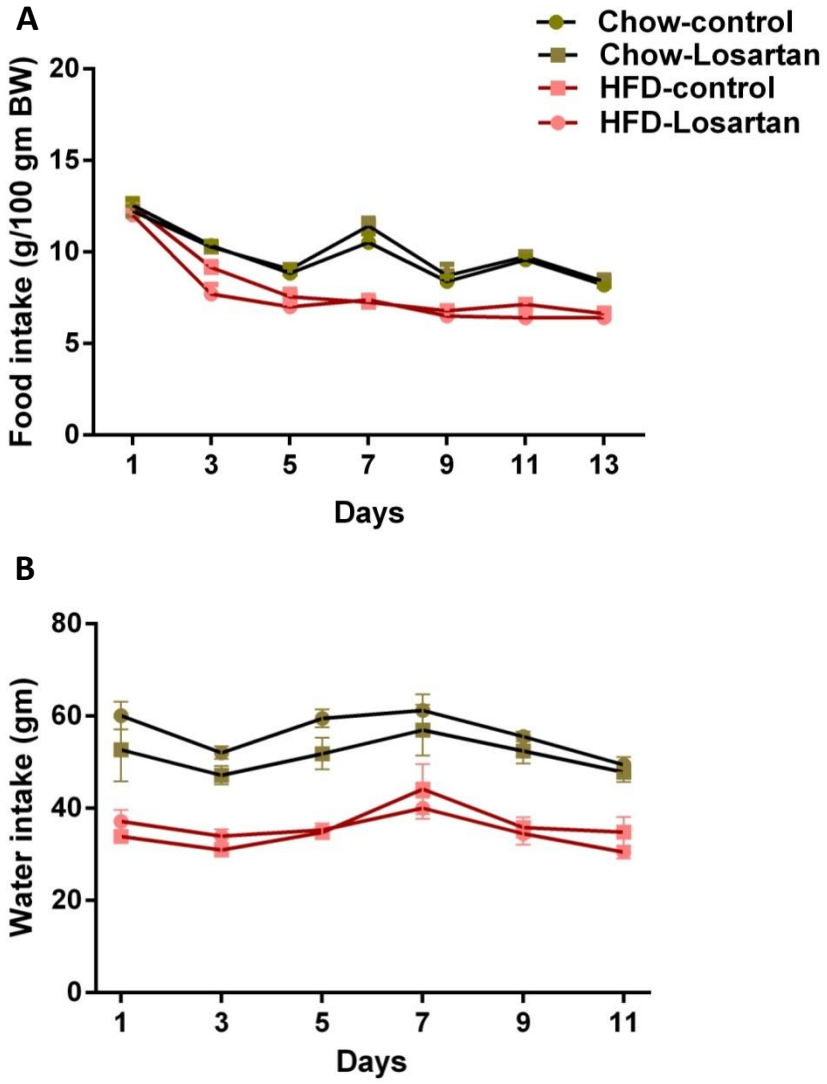


Figure 4.22: **Daily food and water intake of chow and HFD fed rats with or without losartan treatment.**

Line graphs showing mean daily food intake/100 gm body weight (A) and water intake (B) of four groups; rats fed standard rat chow and drinking plain water (chow-control), rats fed standard rat chow and drinking water containing losartan (30mg/kg/day; chow-losartan), rats fed HFD and drinking plain water (HFD-control) and rats fed HFD and drinking water containing losartan (30mg/kg/day; HFD-losartan). Data are presented as mean ± S.E.M.

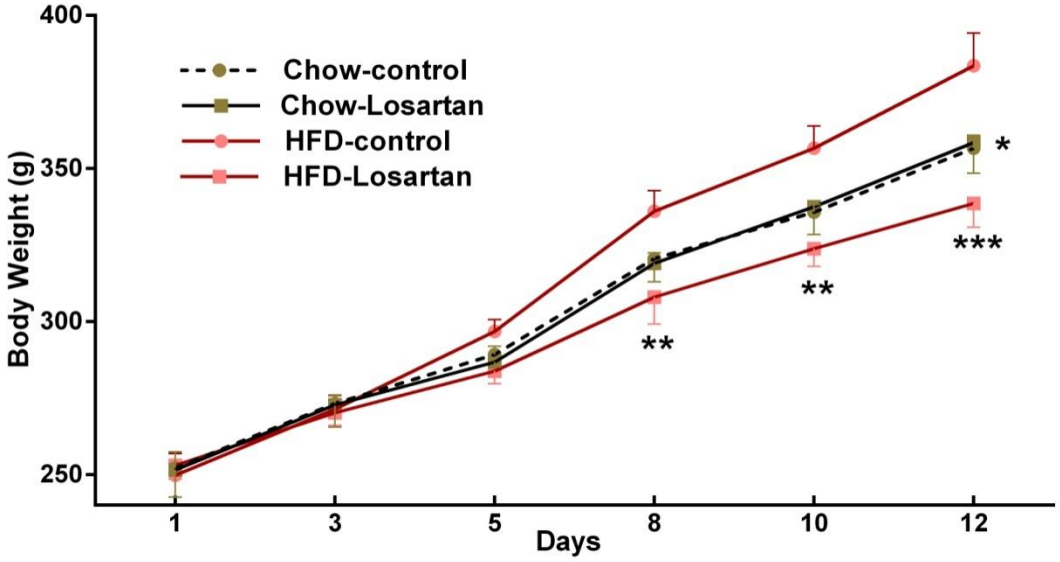


Figure 4.23: **Body weight of chow- and HFD-fed groups with or without Losartan treatment.**

Line graph showing mean body weight of four groups; rats fed standard rat chow and drinking plain water (chow-control), rats fed standard rat chow and drinking water containing losartan (30mg/kg/day; chow-Losartan), rats fed HFD and drinking plain water (HFD-control) and rats fed HFD and drinking water containing losartan (30mg/kg/day; HFD-losartan). *$p<0.05$, **$p< 0.01$ and ***$p<0.001$ represent significant difference from respective HFD-control group. Data are presented as mean ± S.E.M.

4.3.4.2 Fasting blood glucose and obesity indices of chow and HF fed animals with or without losartan treatment.

At the end of the 2 week study, four groups were compared for their fasting blood glucose, body weight/naso-anal length ratio and epididymal/gastrocnemius muscle ratio. The four groups were; rats fed standard rat chow and drinking plain water (chow-control), rats fed standard rat chow and drinking water containing losartan (30mg/kg/day; chow-losartan), rats fed HFD and drinking plain water (HFD-control) and rats fed HFD and drinking water containing losartan (30mg/kg/day; HFD-losartan). As shown in Figure 4.24, there was no significant difference in fasting blood glucose among all four groups. This means that neither HFD-feeding nor losartan treatment affect fasting blood glucose after 2 weeks of feeding or treatment. On the other hand, feeding a high fat diet for two weeks resulted in a significant increase in body weight/naso-anal length ratio compared to rats fed chow (Figure 4.25). Losartan treatment did not affect the weight/length ratio in chow-fed group however, in the HFD-fed rats; losartan caused a significant reduction in weight/length ratio compared to HFD control group (14.4 ± 0.4 versus 16.2 ± 0.4 for HFD-Losartan and HFD-control, respectively). Similar results were observed when comparing epididymal/gastrocnemius muscle ratio (Figure 4.26) where HFD feeding results in a significantly higher ratio compared to chow-fed rats. Losartan treatment resulted in a significant reduction of the ratio in HFD-fed rats with no effect on the muscle/fat ratio in chow-fed groups.

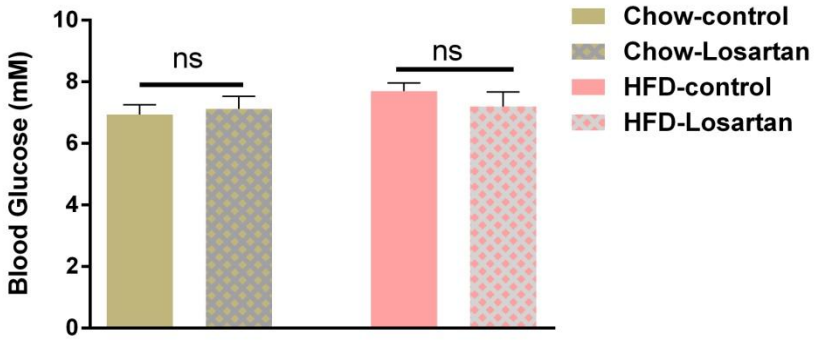


Figure 4.24: **Fasting blood glucose of chow-and HFD-fed groups with and without losartan treatment.**

Bar graphs presenting the fasting blood glucose of the chow-control, chow-losartan, HFD-control and HFD-losartan groups after 2 weeks of HFD feeding. Data are presented as the mean ± S.E.M.

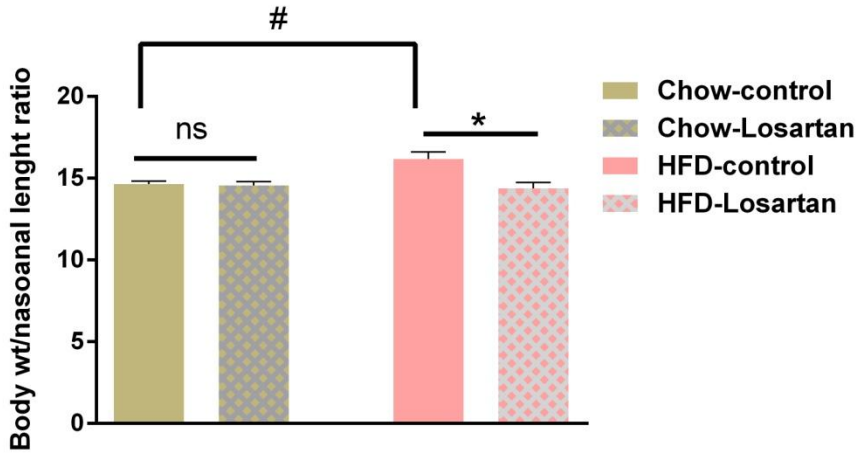


Figure 4.25: **Ratio between body weight and naso-anal length in chow and HFD groups with and without losartan treatment.**

Data are presented as the mean ± S.E.M. *p<0.05 represents a significant difference from HFD-control group and #p< 0.05 represents a significant difference from the chow-control group.

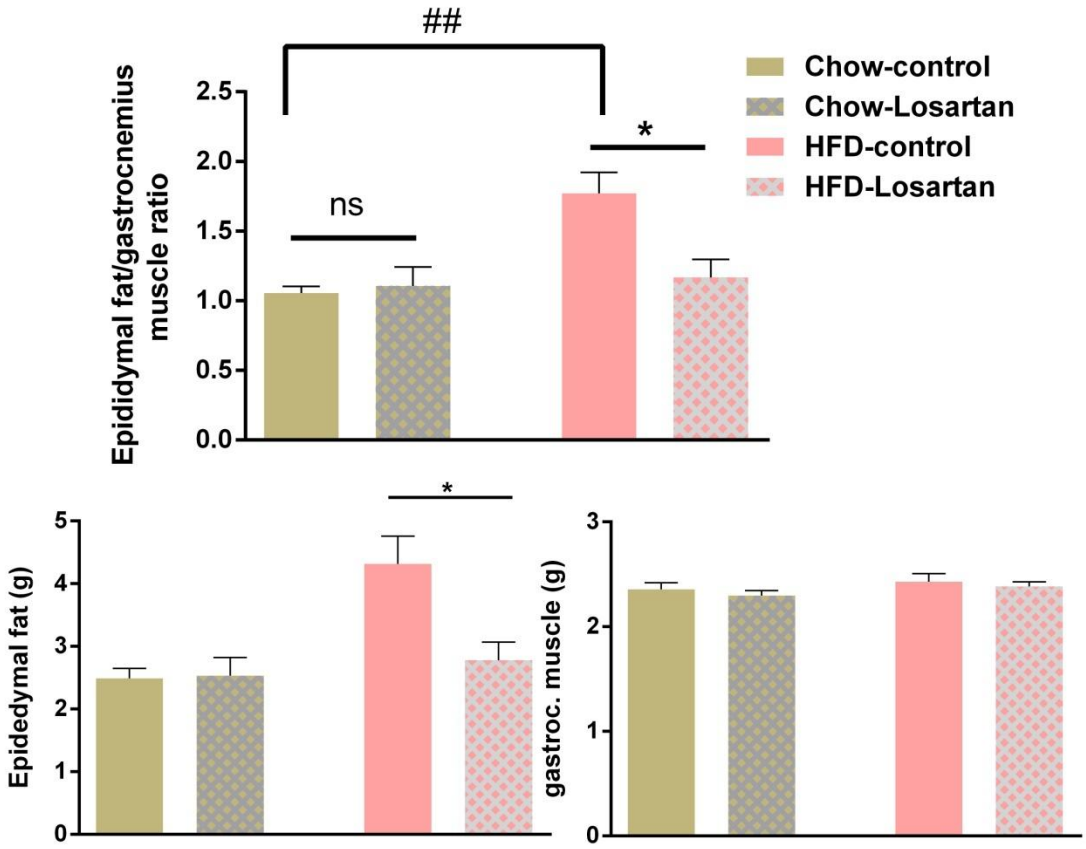


Figure 4.26: **Epididymal fat gastrocnemius muscle ratio in Chow and HFD groups with and without losartan treatment.**

Bar graphs showing the difference in epididymal fat gastrocnemius muscle ratio in the chow-control, chow-losartan, HFD-control and HFD-losartan groups (Upper panel) and individual comparisons of epididymal fat weight and gastrocnemius muscle weights between the four groups (lower panel).

Data are presented as the mean ± S.E.M. *$p<0.05$ represents a significant difference from the HFD-control group and ##$p< 0.01$ represents a significant difference from the chow-control group

4.3.4.3 Effect of HFD with or without losartan on c-Fos immunoreactivity.

As shown in Figure 4.27 A, HFD did not induce an increase in basal c-Fos expression in the AP, compared to the chow-fed group. Losartan treatment did not have any significant effect on c-Fos immunoreactivity in the AP in either HFD or chow-fed groups. In the SFO, and consistent with the results seen in the previous experiments, a significant increase in basal c-Fos expression was observed in the HFD fed rats compared to chow-fed rats (2.0 ± 0.4 neurons versus 36.5 ± 3.3 for chow and HFD-fed groups, respectively; Figure 4.27 A and B). Treatment with losartan resulted in a significant increase in c-Fos immunoreactive nuclei in the SFO of chow-Losartan group compared to chow-control (38.7 ± 2.2 compared to 2.0 ± 0.4, respectively). Combining losartan and HFD resulted in increased in c-Fos expression which was not significantly different from HFD alone. However, it is important to note that the intensity and pattern of c-Fos expression in HFD-losartan is different than what was observed in HFD-control group. As shown in Figure 4.27 and illustrated in Figure 4.28, c-Fos expression in the losartan group appears to be more intense and tends to be more distributed in the inner core of the SFO.

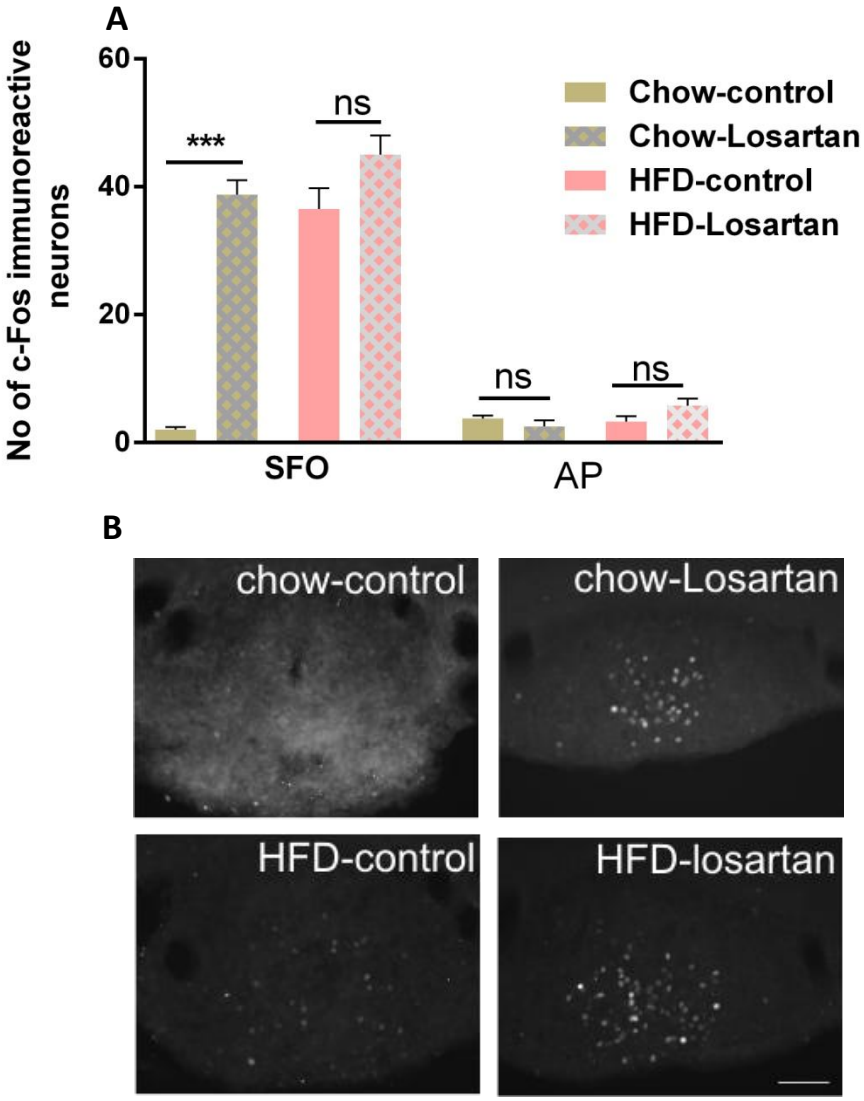


Figure 4.27: **Effect of 2 weeks HFD with or without Losartan treatment on c-Fos expression in the AP and SFO.**

Bar graphs comparing the number of c-Fos immunoreactive cells in the AP and the SFO in chow-fed and HFD groups with or without losartan treatment (A). Data are presented as the mean ± S.E.M, *** $p<0.001$ represents a significant difference from the chow control. Representative immunofluorescence micrographs for all groups in the SFO are shown in B. Scale bar = 100 µm.

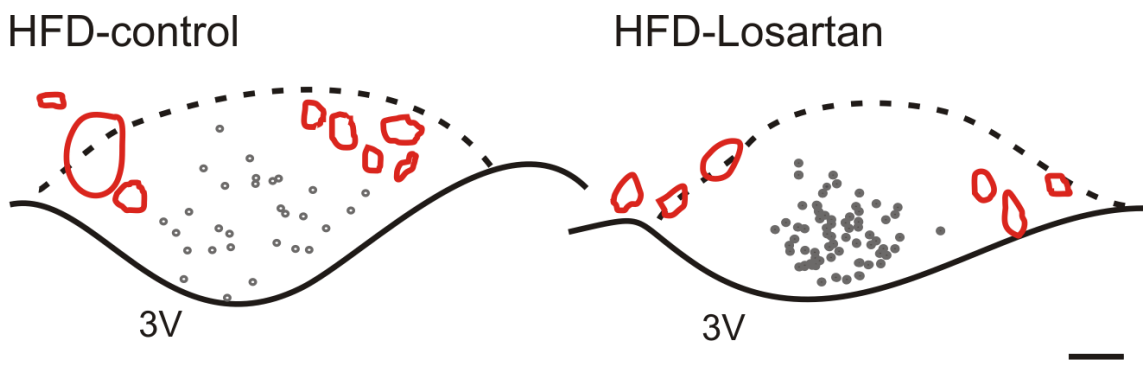


Figure 4.28: Comparison between the pattern of c-Fos expression in response to HFD alone and HFD + losartan.

A diagram comparing the distribution of c-Fos immunoreactive cells in the SFO in response two weeks high fat feeding (HFD-control) and high fat diet feeding combined with losartan treatment (HFD-losartan)**.** Note that c-Fos in HFD-losartan is presented as filled circles which reflects the relative intensity of c-Fos expression.

SFO: subfornical organ, 3V: third ventricle, red outlined structures represent blood vessels. Scale bar = 100 µm.

4.4. Discussion.

The results of the present Chapter show that feeding male Sprague Dawley rats a HFD for 5 weeks resulted in hyperphagia and weight gain. After 4 weeks on HFD, rats started to show variation in the amount of weight gain and could be divided into either diet-induced obese (DIO) or diet-resistant (DR) phenotypes [372, 373]. At the end of the 5 week study, only DIO rats showed increased fasting blood glucose levels when compared to age-matched chow-fed controls. Both DIO and DR rats showed increased weight/length as well as epididymal fat pad/gastrocnemius muscle ratios over chow-fed rats, with this increase being more pronounced in the DIO group. When HFD fed rats were tested for their sensitivity to i.p. CCK, both DIO and DR rats showed a blunted response to CCK in terms of its effect on food intake and induction of c-Fos in the AP and the NTS. Interestingly, in the SFO, basal c-Fos expression was observed in all HFD fed rats, which made it hard to interpret the response of SFO neurons to CCK in the case of high fat feeding.

HFD-induced obesity rats mimic the etiology of common human obesity [391]. As humans do, rats show individual variation in their proneness to obesity. In 1970, Schemmel *et al.* were the first to study individual rat strain variations in their susceptibility to diet-induced obesity [392]. Later, Berthoud *et al.* showed that this susceptibility difference also occurs within a single strain of rats (Sprague Dawley)[393]. In most cases, rats on high fat diet start to show increased body weight over chow –fed controls after 2 weeks on HFD feeding and the obesity phenotype becomes apparent after 4 weeks [338]. This time course is consistent with the results of the present study.

Nevertheless, the time at which these differences start to be evident can vary according to factors like age of animals before starting HFD feeding and type and percentage of fat used [370]. For example, putting rats with approximate starting body weight of 450 gm on a high fat diet containing 32% fat, resulted in a segregated phenotype after 8 weeks of HFD feeding [371]. The actual cause of the different susceptibility to HFD is not well understood, however, it can be partially explained by the better ability of the DR phenotype to adjust caloric intake and reduce their food intake compared to the DIO rats. This was shown in this study and previous work [371, 394]. Moreover, it has been found that DIO rats show reduced leptin sensitivity, which may account for their defense of a higher body weight [395].

In addition to difference in body weight and food intake, DIO not DR rats showed an increase in fasting blood glucose indicative of insulin resistance [396], as well as defective central glucose sensing mediated by sensory neurons [397]. The association between the DIO phenotype and the increased blood glucose has been described before [398, 399]. Body weight/length ratio was used as an index for obesity showing higher values in DIO compared to DR and chow groups. Indeed, there is a number of obesity indices used in the literature, all using body weight and naso-anal length in their calculations. One commonly used one is the Lee index [400], the cubic root of body weight in grams divided by the naso-anal length in mm x 10^4, however, some studies showed that the use of Lee index does not give an advantage over the simpler weight/length ratio [374, 401]. A more reliable index for obesity is measuring adiposity by measuring fat content. Here, I chose to dissect and measure the weight of the

epididymal fat pad, since it has specific and discrete boundaries making its removal feasible and reliable. The weight of this fat pad can be used as a function of the number of fat cells which reflects the degree of adiposity [402]. All the aforementioned measured parameters are markers for the successful induction of obesity for the purpose of studying response to CCK in this model.

When HFD and chow-fed rats were tested for their sensitivity to CCK in inducing a reduction of food intake, an abolition of the effect of CCK was evident in both DIO and DR groups, suggesting that this effect is caused by HFD feeding rather than body weight or adiposity. Similar results have been previously described [171, 230, 403, 404]. For example, Covasa and Ritter [171] showed that adaptation to HFD results in reduced satiety in response to CCK. In their study, they used low and high caloric diets with high fat content and compared them to a low fat diet. CCK effects were attenuated in all high fat diets fed groups, regardless of the effect on body weight, thus leading to the conclusion that the reduced response to CCK is due to the fat content of the diet [171].

The abolished food intake response to CCK in HFD fed rats was paralleled by a reduction in c-Fos expression in the AP and the NTS in response to systemic CCK. This effect has been previously reported [352, 405], and is assumed to be as a result of reduced responsiveness of vagal afferents to CCK and reduced expression of $CCKR_1$ in the nodose ganglion [230, 405]. High fat diet results in elevated levels of CCK [286], and studies showed that chronic exposure to CCK resulted in reduced effect of CCK [406, 407]. Thus, HFD-induced increases in CCK might account for the reduced sensitivity to CCK. Regarding the SFO, HFD fed rats showed elevated basal c-Fos expression with

vehicle treatment and when the HFD group was given CCK, c-Fos induction of a similar magnitude was observed in the SFO. Thus, it was hard to conclude if the SFO response to CCK is modified in response to high fat diet.

It has been shown that some SFO neurons are glucose-sensitive, which means they respond to increased circulating glucose levels [161]. Thus, the observed increase in blood glucose in DIO rats might be a possible cause of the basal neuronal activity shown in this group; however, this cannot be applied to the DR group which did not show elevated blood glucose relative to chow controls. The observed increase in Iba-1 immunoreactivity in the SFO of HFD fed rats (both DR and DIO) indicative of microglial activation could be another plausible explanation of this basal increase in c-Fos in the SFO of HFD fed rats.

Increased basal neuronal activity in the form of increased expression of c-Fos has been reported before in similar situations in other brain areas. For example, HFD fed mice had elevated neuronal c-Fos immunoreactivity in the lateral hypothalamus, dorsomedial hypothalamus and perifornical nuclei compared to lean mice [408]. Thus, it was postulated that continuous activation of these areas might be responsible for the difference in body weight between obese and lean mice [408]. Moreover, in the genetically obese ob/ob mouse model, two different groups reported increased basal c-Fos immunoreactivity in the dorsomedial hypothalamus [409] and the paraventricular nucleus of the hypothalamus [410].

This elevated neuronal basal activity in the SFO in the 5 week HFD feeding study lead us to ask if a shorter duration of HFD feeding (2 weeks) will cause the same

phenomenon. The results of the 2 week study showed that rats fed HFD for two weeks gained weight over the chow-fed controls. As expected, the HFD fed rats did not have elevated blood glucose level compared to chow-fed rats, but they still showed elevated obesity and adiposity indices relative to controls, although not to the same extent as we observed in the 5 week study. When basal c-Fos immunoreactivity in the SFO was studied, the 2 weeks HFD-fed rats showed increased c-Fos expression over the respective chow-fed rats, with no observed basal activity in the AP or the NTS. Looking at microglial status using Iba-1 immunoreactivity, microglial activation was still evident in the HFD fed rats, raising the possibility that increased c-Fos might be linked to this observation. A recent study by de Kloet *et al.* [384] described SFO changes in response to 8 weeks of HFD feeding in mice. They demonstrated that mice fed HFD for 8 weeks had a larger number of microglia within the SFO as well as elevated levels of mRNAs for angiotensin type-1a receptor. They postulated that HFD-feeding initiates an angiotensin-dependent increase in inflammatory cells which might contribute to disturbances to the homeostatic systems regulating metabolism and cardiovascular function [384].

In light of evidence from the work of de Kloet *et al.*, the effect of blocking angiotensin II–type 1 receptor (AT1R) using the non peptide antagonist losartan, was investigated, to test if increased angiotensin activity is responsible for this observed basal neuronal activation within the SFO in response to HFD. In this study, the effect of AT1R blockade with losartan on body weight and food intake in the rat fed high fat diet for two weeks as well as in chow-fed rats was assessed. Similar to previous findings [381, 382, 411], oral administration of losartan reduced weight gain in HFD fed rats with

no effect on body weight in chow-fed group. However, a study by Zorad *et al.* [412], using a different AT1R antagonist; candesartan, has shown that the effect of blocking angiotensin receptor is also evident in chow-fed animals. This discrepancy in the results might be caused by the much longer duration of AT1R antagonist administration (18 weeks versus 14 days in this study), the use of a different strain of rat (Wistar Kyoto rats) as well the use of a different antagonist [412]. A role for AT1R in the induction of HFD-induced obesity was evident using AT1R knockout mice which have shown attenuated diet-induced body weight gain compared to their wild type mice [413]. Interestingly, losartan treatment had an effect only on the body weight of the HFD-fed rats with no effect on food intake. This suggests that the effect of losartan appears in cases where elevated angiotensin activity is present, which is not the case in chow-fed control rats. The fact that water consumption was not altered by losartan, suggests that losartan induced prevention of weight gain is not secondary to increased satiety associated with increased water intake.

The exact mechanism by which blockade of angiotensin action attenuates body weight gain is not fully understood, however, possible mechanisms have been proposed by some studies. For example, using a mouse model that does not express angiotensinogen (angiotensin II precursor), Massiera and colleagues reported that absence of AngII effect results in decreased lipogenesis and increased locomotor activity that is responsible for attenuation of body weight gain and might be centrally mediated [414]. In another study using AT1R knock-out mice, it was postulated that increased

energy expenditure evident by increased rectal temperature and oxygen consumption is responsible for attenuating diet-induced weight gain in this mouse model [413].

In the current study, treatment with losartan failed to block basal c-Fos expression in response to HFD feeding. Surprisingly, losartan treated HFD-fed rats showed increased c-Fos expression that was higher in intensity and distributed mainly towards the core region of the SFO, while a low intensity diffuse pattern was observed in HFD-fed controls. Moreover, losartan treatment in the chow-fed controls resulted in an increase in the c-Fos immunoreactivity similar to what is observed in the losartan HFD-fed group. In addition, although statistically insignificant, losartan treatment increased c-Fos immunoreactivity in HFD rats when compared to HFD-controls. Taken together, these results exclude the possibility that AT1R is directly involved in HFD-induced c-Fos activation in the SFO.

Losartan is a well-established AT1R antagonist that has been shown to attenuate angiotensin induced c-Fos [415] . For example, local pre-treatment with losartan before microinjection of AngII into the SFO has been shown to block angiotensin-mediated c-Fos expression [383, 415]. However, in this study, no data was shown for the effect of losartan microinjection alone. Rowland *et al.* examined the effect of systemic losartan on AngII induced c-Fos in the SFO [383]. They studied the effect of exogenous AngII given by different routes of administration, as well as endogenously released angiotensin (induced by polyethylene glycol administration) and in all cases losartan attenuated AngII induced c-Fos expression [383]. The dose they used ranged from 10-30 mg/kg which matches the dose I used in my study. Similar to the former study [415] ,

there was no data to show the effect of treatment with losartan alone on c-Fos immunoreactivity [383]. In addition, losartan was able to block angiotensin induced depolarization *in vitro* rat brain slices [416]. Collectively, these data support the conclusion that HFD induced c-Fos in the SFO is not directly mediated by activation of the AT1R. However, losartan is documented to cross the blood brain barrier [417], and it might exert its effect in other areas of the brain that project to the SFO. The observed losartan-induced c-Fos activation in chow-fed, as well as in HFD-fed rats might be an indirect effect of losartan on remote areas that in turn lead to neuronal activation and c-Fos expression in the SFO, regardless of the used type of diet.

In summary, the results of this chapter provide evidence of the involvement of the sensory CVOs, SFO and AP, in HFD-induced changes in the brain. It emphasizes the effect of HFD on the response of the AP and the NTS to systemic CCK and provides an indirect evidence of the role of the SFO in short and long term HFD–induced effects. Whether the changes in the activity of the SFO neurons is a cause or a result of HFD induced complications is a question that needs further investigations.

Chapter 5 General Discussion

The overall purpose of this thesis was to compare and contrast neuronal activation of two sensory CVOs, namely AP and SFO, in response to the GI hormone CCK, diet and in states of obesity, in order to understand their involvement in brain-gut axis signaling pathways. The CVOs are central to the maintenance of numerous homeostatic functions including cardiovascular, fluid homeostasis and recently, food intake and energy balance. The gut hormone CCK is an important satiety factor, which is involved in short and long term control of food intake and body weight. Although CCK receptors have previously been shown to be expressed in AP and SFO, nothing was known regarding the action of CCK in the SFO. Furthermore, although central changes in response to HFD feeding and obesity are the subject of extensive investigation, the effect of HFD on the activity and the responses of the SFO to CCK has been never been investigated.

5.1 Summary of major findings.

Measurement of mRNA expression levels in the SFO has demonstrated the expression of receptors involved in the regulation of numerous autonomic functions [144]. Whilst many of these have now been investigated and found to be functionally expressed on SFO neurons [86, 149, 152, 155, 158, 418, 419], the presence of cholecystokinin (CCK) receptors was not examined further.

Three goals of this thesis were firstly to study the response of SFO to the satiety signal CCK, while using the AP and the NTS as positive control regions for CCK-mediated effects. The effect of exogenously administered CCK-8 (i.p.) on the activity of the SFO

was examined using the functional activation markers, c-Fos and p-ERK. The CCK receptor subtype mediating the observed effects was determined using selective $CCKR_1$ and $CCKR_2$ antagonists. Overall, it was found that CCK induced activation of SFO neurons, as shown by increased c-Fos and p-ERK immunoreactivity compared to controls. This effect was blocked by pretreatment with a $CCKR_2$ antagonist.

Second, in order to gain a further understanding of the CCK-induced activation of the SFO, we asked if the observed CCK-induced activation of SFO is a result of a direct interaction between circulating CCK and SFO neurons, or is indirectly mediated through a vagal pathway (as a well-established pathway in CCK-induced inhibition of food intake and activation of the AP and the NTS). This was achieved by studying the effect of CCK on the activity of the SFO in rats subjected to subdiaphragmatic vagotomy, using c-Fos as a marker. It was concluded that the effect of CCK on the SFO was not mediated by the vagus, as c-Fos activation in the SFO was still observed in vagotomized rats in response to CCK. Then, we wanted to investigate the effect of endogenously released CCK on SFO activation. The release of CCK was induced using a meal and by non-nutrient mechanisms with a trypsin inhibitor. We demonstrated that a meal induced activation of SFO neurons which was partially mediated by $CCKR_2$ receptor. However, this effect was not observed using non-nutrient induced stimulation of CCK release.

Finally, because CCK is a satiety hormone and plays an important role in the regulation of body weight, the effect of a HFD model of obesity on CCK-mediated SFO activation was examined. In this model, rats were maintained on HFD for 5 weeks after which the actions of CCK were examined. Unexpectedly, we observed an increase in

basal c-Fos immunoreactivity in the SFO in both phenotypes of HFD-fed rats, DIO and DR rats, without any significant effect on the activity of AP or NTS. We then asked if this effect is attributed to obesity or to due to adaptation to high fat diet. Thus, we investigated basal c-Fos expression in rats maintained on HFD for only 2 weeks and surprisingly, increased basal activation of the SFO was still evident. In an attempt to study the mechanism for this basal activity, we examined the effect of chronic treatment with an AT1R antagonist (losartan) based on a recent study showing activated RAS in the SFO in response to HFD. Unfortunately, treatment with losartan was not able to abolish basal c-Fos expression in the SFO. Moreover, combining losartan with HFD or normal rat chow resulted in high c-Fos expression levels.

Overall, the work in this thesis highlights the role of the SFO in mediating the actions of CCK. It has provided the first evidence of the responsiveness of SFO to circulating CCK from exogenous or endogenous sources. It also supports the possibility that this effect might be through direct interaction of CCK with SFO neurons, as it is unaffected by vagotomy. The data presented in the HFD studies suggested HFD-induced changes in SFO activity, having the potential to be involved in the pathophysiology of obesity, as the effects observed occur before the development of obesity.

Comparison between the responses of AP and SFO.

During the work of this thesis, the main focus was to the study of the response of the SFO to CCK, using the AP as another sensory CVO to serve as a “positive control” region for the action of CCK. The following points compare and contrast the effects of CCK on these two CVOs:

- When the effect of i.p CCK was examined both regions showed increased c-Fos expression over vehicle treated rats, however, the CCK receptor subtype mediating the observed neuronal activity was different. In the case of AP, both CCKR1 and CCKR2 antagonists significantly attenuated c-Fos, in the SFO, however, only CCKR2 antagonism reduced c-Fos in response to CCK.
- Subdiapharagmatic vagotomy resulted in attenuation of CCK effect on the AP while the effect on the SFO was preserved.
- When the effect of meal-induced CCK was examined, both regions showed increased c-Fos expression. However, only CCK-induced c-Fos in the SFO was partially blocked by $CCKR_2$ antagonist, while c-Fos in the AP was not attenuated by any of the antagonists.
- Induction of CCK release by soybean trypsin inhibitor resulted in the induction of c-Fos in the AP only.
- HFD-induced obesity resulted in a blunted CCK response in the AP and an increased basal c-Fos expression in the SFO.
- Short term HFD feeding resulted in elevated basal c-Fos in the SFO only.

The implications of the differences between the response of the AP and the SFO described above will be discussed in the following points:

- The observed difference between the CCK receptor subtype mediating CCK-induced c-Fos and p-ERK in the AP and the SFO is highly suggestive that the physiological output of CCK in response to its activation of the AP is different than that resulting from its interaction with the SFO. Studies showing efferent pathways

originating from the AP and SFO provide a support for this hypothesis. Efferent projections from the AP are mainly to brainstem regions such as NTS, DMV and parabrachial nucleus [420] while, as outlined in Chapter 1, the SFO sends projections to hypothalamic nuclei such LH and PVN [144].

- The dependence of AP activation in response to CCK on an intact vagus, plus the location of the AP in close proximity to the NTS, is indicative that the observed neuronal activation might not be mediated by the direct action of CCK on AP neurons, but more probably due to an indirect activation through the vagus nerve and the NTS. Two observations from the work of this thesis can be used to support this hypothesis: 1) the observed AP activity in response to SBTI-induced release of CCK and the lack of response of the SFO in the same situation and 2) the activation of the AP as well as the SFO in response to meal-induced CCK release which was only blocked by $CCKR_2$ antagonist in the SFO, but not affected by any of the receptor blockers in the AP. In other words, as discussed in Chapter 3, the action of SBTI was suggested to cause local release of CCK that is enough to activate vagal afferents, hence activating the AP and the NTS, but not enough to cause high CCK circulating levels to reach the SFO and cause its activation. In the case of meal-induced activation of the AP which is not dependent on CCK, Fraser *et al.* [421] , reported the same effect and suggested that it is due to the effect of CCK on vagal afferents to sensitize them to other feeding related stimuli such as gastric distension. This can be supported by the fact that a single gastric afferent is multimodal (i.e. responds to CCK and gastric distension) [422] as well as the data

described in Chapter 3 showing a lower number of c-Fos immunoreactive neurons in the AP in response to SBTI (local CCK effect only) compared to c-Fos in the AP in response to a meal (CCK sensitizing the vagus to other meal related stimuli).

- The observed basal c-Fos in the SFO in response to high fat diet and its absence in the AP imply a possible opposite response of these two CVOs to high fat feeding, demonstrated as desensitization to the effect of CCK in the AP and increased basal activity in the SFO. The fact that SFO effects occur early in response to a high fat diet, they may be related to the central changes that occur which ultimately lead to the development of obesity.

Finally, the work of this thesis suggests that the two sensory CVOs (AP and SFO) are important for the actions of CCK and are essential for an integrative response to CCK. We think that the actions of the AP and the SFO might not be overlapping or implying redundancy, but they are important for a complete response to CCK to affect various autonomic functions.

5.2 Key questions and future directions.

One important question raised by the data from this thesis is, what is the physiological output resulting from the interaction of CCK with SFO neurons? As we have done an experiment where we dissected the vagus nerve to test the effect of CCK on food intake, we demonstrated a loss of CCK-induced inhibition of food intake, but a similar magnitude of activation of the SFO. Thus, it is obvious that the effect of CCK on the SFO is not directly related to the satiety effect of CCK, at least in the experimental

paradigm used. Nevertheless, a number of possibilities exist for an action of CCK on the SFO.

One possible action might be related to a satiety effect of CCK which is related to a specific physiological condition. In other words, loss of appetite that is not related to the process of feeding, such as sickness-induced [423-425] or dehydration induced anorexia [426-428]. These types of anorexia are often referred to as adaptive-anorexia [429]. The role of a satiety signal in these situations requires careful assessment and induction of the respective pathological condition. Nesfatin-1, an example of a satiety hormone recently found to be acting on the SFO [150], has been suggested to be a crucial mediator of dehydration-induced anorexia [430]. In fact, nesfatin-1 is an interesting example, as it was found that CCK-8 activates nesfatin-1 neurons in the PVN and in the NTS [198], raising the possibility that CCK might be exerting similar effects in the SFO. Another example of a hormone mediating a form of adaptive anorexia is leptin. A recent study found that leptin is a circulating mediator of LPS-induced anorexia and fever [431]. In order to test this idea, future experiments utilizing models of adaptive-anorexia (such as LPS-induced sickness-anorexia or water deprivation-induced dehydration anorexia) can be used to test: 1) response of SFO to these challenges and 2) ability of one or more CCK receptor antagonists to block responses in the SFO.

As the SFO is known for its role in osmoregulation [432, 433], the action of CCK on the SFO could also be related to an osmoregultory effect of CCK. This idea is supported by evidence from previous studies showing that increased c-Fos [434], as well as p-ERK [435] activity, within the SFO is contributing to osmoregulatory mechanisms.

Interestingly, both activation markers have been observed, in the work from this thesis, to be increased in the SFO in response to CCK. Moreover, a study by Chakfe and Bourque [436], demonstrated that CCK (through a $CCKR_2$-dependent pathway) is involved in the responsiveness of osmoregulatory neurons within the hypothalamus. This could be examined using electrophysiological studies of the SFO using slice preparations to test its responsiveness to changes in bath osmolality in the presence and absence of CCK as well as CCK receptor antagonists.

A role of CCK in temperature control has also been suggested from previous studies [437-439], where it was demonstrated that icv injection of CCK caused a hyperthermic effect through its action on $CCKR_2$. It is important to note that CCK has an opposite effect in the periphery, as it causes hypothermia possibly mediated by its action on peripheral sites [440]. Interestingly, a study showed that the effect of CCK on central control of temperature is not abolished by vagotomy [441]. As described in this thesis, the effect of CCK on the SFO was also not abolished in rats subjected to subdiaphargmatic vagotomy, raising a possibility that CCK might be mediating similar effect in the SFO. Moreover, a study by Weiland *et al.* [442] demonstrated that mice lacking $CCKR_2$ receptors experience blunted response to LPS induced sickness responses including fever, reductions in body weight and food intake emphasizing the role of $CCKR_2$ receptor in these behaviors. Future studies using microinjection techniques to deliver $CCKR_2$ antagonist into the SFO and examine the hyperthermic effect of icv injection of CCK will permit us to test this idea.

Water consumption is another behavior that has been associated with the integrative function of the SFO [68, 86, 415]. In a study using $CCKR_2$ receptor null mice, increased water consumption was observed [252], raising the possibility that the SFO may act as a site mediating effects on drinking behavior mediated by action of CCK on $CCKR_2$. Examining the effect of selective ablation of CCKR-expressing neurons in the SFO on water consumption will provide a direct evidence for this hypothesis. Selective ablation can achieved via microinjection of CCK-saporin (SAP). SAP is a ribosome inactivating protein that if conjugated to CCK, eliminates cells expressing CCK receptors [443].

In addition to the aforementioned effects, CCK exerts a variety of physiological functions that have been associated with the process of meal termination [254]. CCK causes splanchnic vasodilatation that is followed by depressor and bradycardiac effects [255], inhibits renal sympathetic vasomotor nerve activity [256], resulting in increased blood flow to the gut and kidney to enhance the process of digestion and to deal with the additional fluid load following a meal. Thus, the SFO may also represent an additional site in the CNS involved in the cardiovascular effects of CCK, a suggestion supported by the established roles of SFO in cardiovascular regulation [62, 257].

Collectively, the literature described above emphasizes the multifunctional aspect of a single hormone which greatly depends on the type of receptor and the tissue expressing this receptor. A phenomena which increases our understanding of the physiological role of gut hormones, as well as the impact of their deficiency or over-expression, on different systems within the body.

Another important point highlighted by the work of this thesis is the integrative role of sensory CVOs. It was demonstrated that the SFO neurons respond to CCK from exogenous as well as meal-induced endogenous sources. In case of the meal-induced SFO-activation, the effect of $CCKR_2$ receptor blockade was partial reflecting the involvement of other postprandial signals in SFO activation. Thus, this thesis adds further evidence for the function of the SFO as an integrative central site within which a single neuron is able to sense and integrate the critical information from multiple circulating signals (reviewed in details in Chapter 1) and generate an output to control various autonomic functions.

A surprising observation within the work of this thesis was the induction of c-Fos immunoreactivity in the SFO by two antagonists acting in two different receptors namely, devazepide (acting on $CCKR_1$) and losartan (acting on AT1 receptors). We are aware of one similar observation regarding the induction of c-Fos in supraoptic nucleus, dorsomedial hypothalamus and the medial magnocellular subdivision of the hypothalamic paraventricular nucleus by devazepide, where no clear explanation was provided by the authors [129].

Nevertheless, there is a possible explanation that might help understanding the effect of these different antagonists, which was specific to the SFO, based on the reported presence of GABAergic interneurons in the rat SFO [444]. The actions of these antagonists on these neurons might inhibit the release of GABA and thereby remove GABA mediated inhibitory inputs to other neuronal populations. This could be a plausible explanation for the observed c-Fos expression. In support of this idea, there is

a study that reported the action of CCK-8 on GABAergic interneurons in the amygdala resulting in increased inhibitory transmission [249] and another demonstrating the co-localization of AngII and GABA within rats SFO [445] and suggesting a modulatory effect of AngII on the release of GABA. Future electrophysiological studies on SFO slice preparations examining the effect of direct application of these antagonists, with or without GABA, application on the excitability of SFO neurons will provide evidence to support this suggestion.

Another key question raised by the work of this thesis is the effect of HFD-induced obesity and adaptation to HFD (for a period of two weeks) on the activity of SFO neurons. Interestingly, this effect was specific to the SFO and was not observed in the AP. Based on the fact that SFO is a site of action of AngII, and the reported increased RAS activity associated with high fat diet [384], we asked if blocking the effect of AngII on the SFO using losartan in HFD fed rats will reduce the observed elevated basal c-Fos expression. The answer we found was that it was probably not mediated by the action of AngII, at least in a direct way. Nonetheless, this observed basal activity emphasizes the role of SFO in metabolic control. A similar increase in basal c-Fos in response to HFD was reported in other brain regions such as the LH and DMH [408]. LH and DMH are hypothalamic regions known to be involved in the regulation of energy balance (as discussed in Chapter 1).

Increased endoplasmic reticulum (ER) stress was reported in the SFO in response to HFD and was associated with HFD-induced hepatic dysfunction [446]. ER stress occurs as a result of stress signals such as disturbances in cellular redox regulation and the

production of reactive oxygen species, hyperglycemia, and hyperlipidemia which alter ER homeostasis making it dysfunctional. ER stress is thought to play a role in diseases such as atherosclerosis, diabetes and provides upstream signals to the induction of c-Fos expression [447]. Increased ER stress in the SFO has been found to be associated with AngII induced hypertension [448](a common complication of obesity). Thus, increased ER stress within the SFO can serve as a plausible explanation for the observed c-Fos induction. In addition to ER stress, the increased microglial activation discussed in Chapter 4, can be linked to basal c-Fos activation. Increased basal activity of the SFO highlights the importance of SFO as a central site affected by HFD and emphasizes its role as a CNS region with integrative functions. Nevertheless, the reason of this increased c-Fos remains a subject for further investigation.

5.2 Concluding remarks.

The role of the SFO is increasingly recognized as a target for the actions of a number of circulating signals involved in the regulation of autonomic functions. The SFO has a potential role in energy balance supported by the expression of different receptors for signals involved in controlling energy homeostasis has extensive neural projections to hypothalamic areas with well studied roles in energy homeostasis.

In conclusion, the data presented in this thesis suggest that the direct actions of CCK on the SFO. The SFO is an ideal target for CCK due to the lack of the BBB and the ability to monitor the contents of the circulation. This information can then be transmitted via well-established projections to autonomic nuclei in the brainstem and hypothalamus, providing a route by which the circulating satiety signal, CCK, can act to

regulate a variety of physiological functions. In addition, the work of this thesis introduced the SFO as a potential contributor in the pathophysiology HFD-induced obesity.

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