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DEFICITS IN FEEDING AND VAGAL RESPONSES IN TWO RAT MODELS OF OBESITY

A Thesis in

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by

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ABSTRACT

Obesity is currently a global epidemic, while in the United States, approximately 33% of the population is obese. Consumption of a HF diet is linked both with overconsumption and obesity in humans and rodents. Cholecystokinin (CCK) a peptide hormone, secreted from the duodenum in response to intestinal fats and proteins, is partially responsible for meal termination. While a HF diet suppresses the satiating effects of CCK, fats also exert orosensory stimulating effects, which promotes further intake of both fats and other nutrients. Because obese individuals have increased preference for fats compared to lean individuals, it is possible that increased orosensory stimulation causes over consumption. The Otsuka Long-Evans Tokushima Fatty (OLETF) rat is a model of obesity that lacks a functional CCK-1 receptor (CCK-1R). This rat is hyperphagic compared to Long-Evans Tokushima Otuska (LETO) lean controls. Therefore, in an attempt to elucidate the role of the orosensory component in hyperphagia in the OLETF.rat, we employed one bottle sham feeding tests. To examine the role of an energy deficit in preferences of fats, we subjected OLETF and LETO rats to non-deprivation, overnight deprivation, and two hour deprivation and tested their acceptance for fat. Additionally, to examine the interaction between both the oral and postoral properties of fats, we assessed acceptance and preference for fats using real feeding tests. We found that OLETF rats' sham intake was significantly increased at higher oil concentrations during a fed state compared to LETO. Conversely the concentration curve was

shifted left towards the lower concentrations after deprivation. Real feeding also resulted in increased oil intake at high oil concentrations compared to LETO. Together, these results suggest that OLETF rats have an increased oral avidity to fats compared to LETO. During deprived conditions, OLETF consumed more from low concentrations of oils. To examine bodyweight and adiposity as well as peripheral deficits in an established rodent model that becomes obese when fed a high-fat (HF), we measured 24-hour chow intake, body weight, and relative fat pad mass in dietary induced obese (DIO) and dietary induced obese resistant (DR) rats. Additionally, we studied the sensitivity of both DIO and DR to CCK at doses of 2.0, 4.0, and 8.0 μg/kg when fed chow over 60 minutes. Finally, to complement our behavioral experiment, we examined CCK-induced Fos-like immunoreactivity (Fos-Li) in the dorsal vagal complex (DVC) after pre-treatment with 4.0 and 8.0 μg/kg doses of CCK. We found that DIO rats ate more chow in 24 hours and weighed more than DR rats. However, epididymal and total relative fat pad mass was significantly lower in DIO rats compared to DR. Lastly, CCK significantly suppressed chow intake more and produced more Fos-Li in the DIO strain compared to DR at the 4.0 μg/kg dose, These results show that DIO rats are more sensitive to the satiating effects of CCK at 4.0 μg/kg that is most likely due to vagal mechanisms, such as an increase of CCK-1R.

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COMMON ABBREVIATIONS

AP – **area postrema**

CCK – **cholecystokinin**

DIO – **diet induced obese**

DR – **diet induced obese resistant**

DAB – **diaminobenzidine**

DMV – **dorsal motor nucleus of the vagus**

DVC – **dorsal vagal complex**

Fos-LI – **fos like immunoreactivity**

GI – **gastrointestinal**

IP **– intraperitoneal**

LETO – **long-evans tokushima otsuka rat**

NTS – **nucleus tractus solitarius**

OLETF – **otsuka long-evans tokushima fatty rat**

rmANOVA **– repeated measures analysis of variance**

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CHAPTER 1

GENERAL INTRODUCTION

Disordered satiation in dietary and genetically obese models

Obesity is a phenotypic trait expressed by a variety of rodent strains, including rats and mice with spontaneous genetic mutations, and mice with experimentally produced gene deletions (4). Remarkably, obesity is a concomitant of alterations in a surprising variety of disparate genes. A short and non-exhaustive list includes deletions or mutations of leptin receptor genes, the GRP receptor gene, the 5-HT2c receptor gene, and the CCK-1 receptor gene [for review see (4, 6)]. The variety of mutant and transgenically modified alleles associated with obesity probably reflects the complexity and diversity of processes that impinge on control of body energy balance and the number of points at which the balance can be disturbed.

Otsuka Long Evans Tokushima Fatty (OLETF) rat

The OLETF rat is an outbred strain of Long Evans rats, which has been established as an animal model of non-insulin-dependent diabetes mellitus and obesity (52). This strain has a mutation lacking expression of the CCK-1 receptor

gene (108). Consistent with this mutation, little or no CCK-1 receptor mRNA is detected in the pancreas of the OLETF rat (35). Furthermore, pancreatic acini from OLETF rats are completely insensitive to stimulation of enzyme secretion by CCK. This absence of CCK sensitivity is specific in that OLETF acinar cells do secrete amylase in response to bombesin, carbamylcholine and secretin. Also, pancreatic CCK binding in OLETF rat is completely absent (78).

OLETF rats exhibit accelerated rates of weight gain, compared to control rats (72, 94). Beginning at 5 wk of age, and results in ~40% increased body weight compared to the control Long Evans Tokushima (LETO) strain. As they become obese, OLETF rats gradually develop hyperglycemia, hyperinsulinemia, and non-insulin-dependent diabetes (NIDDM), which is well established by ~ 18 wk of age (52). Plasma glucose levels following oral glucose loads are elevated as early as 8 weeks of age and are significantly impaired by 24 weeks. OLETF rats are less sensitive to within-meal satiety signals and this has been attributed to their lack of CCK-1 receptors. For example, systemic administration of CCK has no suppressive effect on food intake (21) or gastric emptying (101) in OLETF rats. OLETF rats ingest larger meals and are less sensitive to inhibition of food intake by some intraintestinal nutrients than LETO control rats (21, 72). Furthermore, OLETF rats become resistant to peripheral leptin injections (76). Because of the reported synergistic interaction between CCK and leptin on food intake and body weight (30), defects in CCK signaling could have long term consequences on body weight control. Such a possibility is supported by recent work with OLETF rats. Dr. Covasa's prior work indicates that OLETF rats exhibit

a broad range of satiation deficits (21). However, the work with CCK receptor antagonists in OLETF and LETO rats suggests that not all OLETF satiation deficits are directly related to absence of CCK receptors.

CCK-1 receptor deficiency and hyperphagia

The underlying cause(s) of hyperphagia in this rat model has not been fully revealed. Evidence so far, suggests that the absence of CCK-1 receptors and alterations in peripheral and central CCK signaling remain the most likely explanation. This is based on data showing that these animals exhibit a range of deficits in meal related satiation signals such as CCK and intraluminar nutrients (21). More recently, our laboratory showed that increased food intake in obese OLETF rats can be attributed, in part, to altered orosensory functions, i.e., increased preference for high concentrations of sweet solutions (26) and heightened reward sensitivity (45). In addition, obesity as well as NIDDM can be greatly reduced by caloric restriction (77) or exercise (8) suggesting that obesity and NIDDM in OLETF rats with CCK-1 receptor deficits are secondary to their hyperphagia. However, overall hyperphagia indicates that OLETF rats may have additional deficits beyond that of controlling meal size such as those involved in the control of energy balance. In this regard, OLETF rats have been shown to have an intact leptin signaling system (72), suggesting that a deficit in this system is unlikely to account for their hyperphagia and obesity. Therefore, it becomes obvious that additional CCK- and/or non-CCK deficits may be responsible for OLETF rat’s chronic hyperphagia.

Altered motivation in OLETF rats

During physiological need-states, such as food-, or sodium-deprivation (27, 88, 89) or limited access of palatable food [e..g. sweet or fatty meals: (44)] results in increased intake when the stimuli become available again (i.e., “reward sensitization”) (see (15) for a review). For example, OLETF rats overeat on meals that are normally preferred [fat: (94); sugars: (26)], However, whether OLETF rats prefer fats more than LETO rats based on their orostimulatory effects is not known. Sham feeding provides a measure of orosensory stimulation in the absence of significant postingestive negative feedback because it minimizes the stimulation of gastric nutrient receptors, eliminates the stimulation of gastric distention receptors, the role of gastric emptying, and minimizes or eliminates the stimulation of intestinal and postabsorptive receptors (41, 100). Rats sham ingest sucrose and lipid emulsion in amounts that increase monotonically with concentration (41, 74, 115). In the obese rat the hyperphagia for fats seems to be most related to the positive feedback from orosensory properties. Of direct relevance to this thesis is that suppression of food intake by both sugars and lipids is mediated by CCK-1 receptors [see (84), for review]. OLETF rats are less sensitive to the satiation effects of intragastric and intraintestinal infusion of glucose and lipids (21, 94). However, while LETO rats reduce food intake in response to glucose infusion at both 30 min and 4 h postinfusion, OLETF rats reduce intake during the first 30 min, but intake is no longer reduced by 4h postinfusion. This argues for the participation of orosensory components in

glucose-induced reduction in sensitivity in OLETF rats. In the current work lipid as stimuli was chosen because 1) it is highly preferred by rats; 2) is most suitable for experimental dissection of orosensory and postingestive determinants; 3) promotes hyperphagia, a necessary condition to probe the relevance of orosensory and postingestive contribution to overeating; 4) is one main representative of macronutrients class; 5) increased fat intake is parallel with increase in obesity.

Oral stimulation by fats

Both orosensory properties and postoral nutritive responses contribute to the reward quality of food. The orosensory properties of fats stimulate ingestion. This occurs in deprived and sated sham-feeding animals for nutritive (corn oil) and non-nutritive (mineral oil) stimuli (1, 69). Thus, ingestion of fats occurs in the absence of feedback with regard to energy status. Orosensory stimuli such as fatty flavors are rewarding, and they promote approach behavior and operant learning even when postoral feedback is minimized (96).

Oral fat exposure initiates cephalic phase responses throughout and beyond the GI tract (gastric lipase secretion, GI transit.pancreatic secretion, gut hormone release). Dietary lipids in the mouth may promote ingestion while its later presence in the intestine alters gustatory coding possible leading to reduced appeal and prompts release of satiety hormones to terminate ingestion. The postingestive actions of nutrients on food intake are usually considered to be

inhibitory, but recent work demonstrates that nutrients can have positive postingestive effects that can increase food preferences and acceptance (97). Thus, hyperphagia induced by the postingestive actions of HF diets may result from increased positive feedback as well as, or instead of, decreased negative feedback. It is clear that both oral and post-oral influences are important contributors, however, the role of chemosensory input pre- and post-ingestively in the control of fat intake is poorly understood.

Participation of cholecystokinin (CCK) in satiation

Gastrointestinal satiation peptides such as CCK exert control on a variety of functions that control ingestion (83). The peptides are secreted by peripheral tissues and organs, they all reduce food intake by reducing meal size when administered exogenously, are secreted in response to nutrients, and interact with nutrients to reduce intake (84). The signals that ultimately cause satiety can be generated in the peripheral nervous system and relayed to the brain where they become integrated with other determinants of meal size. Defects in the functionality of peripheral satiation signals result in alterations in short term food intake as well as in long term signals leading to obesity. Gibbs, Young and Smith (38) discovered that exogenous administration of CCK causes a dose-dependent decrease in meal size. Subsequent studies have defined CCK as one of the most biologically potent satiety peptides. CCK reduces food intake by acting on vagal sensory neurons (71). Surgical (82) as well as chemical (85) destruction of vagal

sensory fibers abolishes CCK-induced reduction of food intake. Both systemic capsaicin (105) and nodose ganglionectomy (53) reduce CCK binding in the nucleus of the solitary tract, the site where vagal sensory fibers terminate. Furthermore, CCK binding sites (receptors) are transported by vagal sensory neurons (73). CCK receptors have been identified and the genes coding for these receptors have been cloned (28). Finally, results of electrophysiological experiments indicate that vagal sensory fibers innervating the gastrointestinal tract can be activated by exogenous CCK (9). Studies using selective CCK-1 and CCK-2 receptor antagonists indicate that reduction of food intake by injections of exogenous CCK is mediated by CCK-1 receptors (14).

Reduction of food intake by exogenous CCK is reported to be attenuated or absent in at least two obese rat strains (67), as well as in lean rats (22) and people that are fed a high fat diet (34). Thus, there is convincing evidence to assume that obesity is associated with reduced efficacy of CCK.

Vagal transmission of peripheral satiation signals leads to neuronal activation of hindbrain structures.

Immunohistochemical detection of c-Fos expression is a useful marker for neuronal activation and tracing polysynaptic pathways in central and peripheral sensory neurons. Short term stimulation of nerve cells is followed by transcriptional and translational activity of selected genes including the c-*fos* oncogene, resulting in the production of intracellular regulatory factors like Fos protein. A number of anorectic signals, including CCK, have been shown to

increase the expression of c-Fos in the dorsal medulla as a result of vagal nerve transmission (25, 33, 66, 86). CCK-1 receptors are necessary for communication of CCK-induced vagal signals from the gut to the dorsal hindbrain (21, 91, 92). This is most evident by the fact that communication of these signals is impaired in rats that do not express CCK-1 receptors (21, 72). In the current thesis, I intended to examine whether CCK-8 administration results in a deferential response in obesity prone versus obesity resistant animals.

Dietary obesity

A growing number of reports indicate that obese subjects, including humans, exhibit disruptions in their responses to satiation signals. For example, obese humans reported feeling less hungry than lean subjects (34, 36, 68), and they were less sensitive to infusion of gastrointestinal peptides such as bombesin (63). Rats made obese by overfeeding also were less sensitive to the satiating effects of CCK (111).

The DIO: a rat model of HF diet-induced obesity

A number of genetically obese models have been developed [for a review, see (110). However, such monogenic obesity syndromes are rare in human, and most human obesity represents the interaction between multiple genes and environment, of which diet is a major component. Similar to humans (64), rats are also attracted to fat and overeat when placed on a hypercaloric HF

diet and become obese (37). Consequently, polygenic rodent models of diet-induced obesity are being subjected to increasing attention. One such model is the outbred Sprague Dawley (SD) rat. It is characterized by variability in susceptibility to obesity on a high energy HF diet (59, 60), a situation that has resonance in the human population. HF diets, due to their high palatability and energy density, stimulate voluntary energy intake. An increased fat intake does not stimulate its own oxidation but the fat is stored in the adipose tissue (32). Even when diet composition is isoenergetically switched from low to HF, fat oxidation only slowly increases, resulting in positive fat balances on the short term (93). Rats that are adapted to HF diet also express an increased appetite for fat. Mechanisms underlying this persistent change in the metabolic and motivational regulation of food intake are not fully understood. It has been suggested that prolonged exposure to HF diet may result in impaired gut-brain signaling (90). Thus, it is plausible that the alteration in preference function and reward sensitivity, at least in part, is due to maladaptive feed back mechanism that develops in response to sustained overconsumption of HF diets in obese individuals.

When outbred SD rats are placed on an energy dense, HF diet, there is a wide distribution in body weight gain; a subset of animals becomes very obese (DIO), whereas others remain as lean as animals fed a low-fat diet [diet-resistant (DR)] (61). Prior work in DIO rats has shown that hyperphagia and increased energy efficiency often accompany the persistent obesity produced by long-term,

HF feeding (58). The underlying mechanism(s) by which HF feeding produces hyperphagia in this model are not completely known. However, in addition to deficits in responses of hypothalamic systems to dietary obesity (57, 60), there is circumstantial evidence that these rats have decreased peripheral sensitivity to food stimuli (40, 43, 55). In addition, we showed that chronic maintenance on a HF diet leads to decreased sensitivity to some satiation signals and diminished vagal responses (18, 19). Therefore, the work in this thesis aims to determine whether some of the deficits described in the OLETF rat that may be responsible for hyperphagia and obesity in a dietary induced obesity DIO model.

Specific Aims, Experimental Design, and Relevance

Consumption of dietary fat causes changes within the alimentary, metabolic and humoral systems that may promote a more efficient process for energy metabolism from this rich source, and may also lead to the storage of energy in the form of adipose tissue. Although still controversial, some studies in humans show that obese like sugar better than underweight individuals (5). For fats, a link between obese and an enhanced preference for high fat foods has been shown (29). Previously, our laboratory showed that OLETF rats have an increased preference for sweet solutions. Specifically, compared to lean LETO controls, the OLETF rats exhibit increased real and sham intake of normally preferred sucrose solutions with a heightened preference for higher over lower concentrations (1M vs. 0.3M) (26) and these effects were accentuated with the development of obesity and diabetes (46). This led to the suggestion that the

increased meal size seen in the OLETF rats might, in part, be due to their enhanced orosensory stimulation by sweets. Furthermore, there is abundant evidence showing that rats prefer fats based on their orosensory stimulatory effects and that OLETF rats are less responsive to the suppressive effects on fats when present in the intestine. Therefore, **Specific Aim 1 of this thesis was designed to address the hypothesis that OLETF rats also exhibit an avidity for fat consumption,**

The role of gastrointestinal peptides in control of food intake and their potential use as tools in obesity treatment has been an area of intense study for the past three decades. Although experiments using selective CCK receptor agonists and antagonists have not produced compelling evidence that CCK directly contributes to control of adiposity, several lines of evidence lend support for the participation of cholecystokinergic system in long term control of food intake and body weight gain. For example, some genetically obese rodents, such as the Zucker fatty rats, are less sensitive to CCK and other satiation signals from the gastrointestinal tract (67). The OLETF rats, which does not express CCK-1 receptors also overeats and becomes obese (72). Not surprisingly, these mutant rats do not reduce their food intake in response to exogenous CCK and have deficits in satiation in response to other peripheral signals (72). Hence, it may be that some satiation deficits in Zucker fatty rats and OLETF rats result from sequels of the obese or diabetic phenotype, which are not directly or entirely related to reduced CCK receptor function. Furthermore, animals maintained on a HF diet are less sensitive to the suppressive effects of CCK (22, 90). These

animals also show impairment in some gastrointestinal functions as well as decreased vagal sensitivity (18-20). The dietary induced obesity rats (DIO) that resembles human obesity phenotype represents an excellent model to examine potential peripheral satiation deficits. Therefore, **Specific Aim 2 of the present thesis was designed to examine differences in CCK sensitivity between DIO and DR rats as well as the behavioral and neuronal correlates associated with CCK responses.**

Chapter 2

REAL AND SHAM OIL ACCEPTANCE AND PREFERENCE IN THE OLETF AND LETO RAT

Introduction

Substantial amount of evidence has placed the high amounts of fatty foods within the western diet as a cause for the obesity epidemic (80). In animal models, chronic consumption of a HF diet has been shown to increase resistance to the effects of anorexigenic signals, such as cholecystokinin (CCK) (20, 90) and insulin (4). Previous literature has also shown that rats will readily sham feed mineral oil and corn oil emulsions, suggesting that the orosensory component of oils is enough to elicit intake (69, 109). Although rats drink oil emulsions in sham and real feeding, preference for specific tastes depends heavily upon previous exposure (81, 95) and the energy balance both long-term and short term of the rat (65, 98).

Sham feeding is a way to limit intestinal negative feedback inhibition while allowing the study of orosensory components to specific stimuli (24, 117). Previous data affirms that in the case of sham feeding oils, the orosensory component produces positive feedback while intestinal effects exert negative feedback on food intake. During sham feeding, rats drink oil solutions as an inverted U-function of concentration, with a peak at a moderate oil concentration (25% and 50%). Other reports have shown that from 12.5% to 50% concentrations of oil, rats will sham feed oil similarly (69). After sham feeding

oils, the orosensory component is strong enough to elicit a response to stimulate intake of test foods in rats even when non-deprived of food (109), further illustrating the importance of this positive feedback. When sham fed oils during two-bottle preference tests, rats increase preference for corn oil over mineral oil (1, 103). One dispute to this claim is that sham feeding does not completely prevent post-ingestive absorption, which could allow for the intestine to be conditioned to the oils.

The OLETF rat, a rodent model for non-insulin dependent diabetes mellitus also contains a natural single point deletion of the CCK-1R. OLETF rats are hyperphagic compared to LETO controls and become obese. By week 18, the OLETF are hyperglycemic with type NIDDM occurring in week 24 (52), while leptin resistance occurs at week 8 (76). CCK, which is secreted from the I-cells in the duodenum in response to fat and protein, plays a pivotal role in short-term satiation. Thus, the OLETF rat should have less satiation from intestinal feedback elicited from fat intake than LETO controls. This has been supported by Schwartz et. al (94), as well as our work in the OLETF, showing suppression of food intake in LETO, but not OLETF rats 4 hours post duodenal infusion of oleic acid. In addition to peripheral satiety defects, which serve as negative feedback in food intake, the OLETF rat also maintains increased positive feedback compared to LETO controls in orosensory responses to palatable food sources (26, 46).

While the OLETF has increased orosensory sensitivity to palatable food sources (26, 46), infusion of nutrients via duodenal catheter during sham feeding

delays intake of sucrose significantly less in the OLETF compared to the LETO. This provides evidence that the OLETF rat has a decreased intestinal sensitivity as well as increased oral sensitivity to sucrose (26, 46). Also, as well as having deficits in peripheral satiation and enhanced orosensory stimulation, the OLETF maintains altered levels of dopamine, which positively correlate in reward with such palatable sources (2, 45, 47, 48). Additionally, OLETF rats fed a HF diet have decreased intestinal sensitivity to intakes of solid HF foods (94). Although the OLETF has a decreased intestinal sensitivity to fats, the orosensory component of fats in the OLETF rat has not been previously examined.

Measuring orosensory stimuli in rodent models typically involves the use of both acceptance and preference testing in sham feeding. Acceptance testing measures the rats' likelihood of drinking a solution during one-bottle presentation while preference testing measures the rats' desire for one solution over another solution. To measure acceptance, often different concentrations of one or more solutions are given to subjects on different testing days; measuring preference involves presentation of both solutions at a given concentration, usually at the same concentration or same hedonic response level.

Therefore, to examine the orosensory acceptance of oils during deprived and non-deprived states in the pre-diabetic OLETF compared to LETO controls, we sham fed OLETF and LETO rats increasing corn oil emulsion concentrations (12.5, 25, 50, 75, and 100%) under both non-deprived and food deprived conditions. To examine the relationship of the orosensory component with feedback inhibition from the gastrointestinal tract, we also employed real feeding

after food deprivation. We chose to use oil emulsions because rats will more readily consume an emulsion compared to 100% oil. Additionally, to measure preference between the two strains in regard to nutritive and non-nutritive oils, we performed brief access (30 min) two bottle preference tests with 100% emulsions of corn oil and mineral oil.

Methods

Animals

OLETF and LETO rats were donated as a generous gift from the Tokushima Research Institute, Otsuka Pharmaceutical, Tokushima, Japan. Rats were housed individually in mesh floored, hanging steel cages in a temperature controlled vivarium on a 12:12 light:dark cycle (lights on at 0600 hours) and were provided ad libitum access to pelleted rat chow (Purina 5001) and water, except during the night before experimental procedures as described below. Rats were handled for a minimum of 1 week during acclimation period before experimental protocol began.

Surgical Procedure: Gastric Cannulation

Following overnight fasting, rats were anaesthetized with a combination of ketamine (50mg/kg), xylazine (5mg/kg) and acepromazine (1mg/kg). Gastric cannulation was performed as described in Yox and Ritter (118). Briefly, after anesthesia, rats were surgically fit with chronic gastric cannulae on the non-glandular portion of the stomach. A puncture wound was made in the lateral

section of the rat's torso through which the cannulae was passed. The cannulae were then secured with a mesh insert. Rats were allowed at least 2 weeks to recover from the surgery before any feeding experiments began.

Experiment 1: Non-deprived Sham Feeding

Rats (n = 13, 6 OLETF and 7 LETO) were used in sham feeding for non-deprived conditions. In the morning (0900 hours), rats were weighed and their stomachs lavaged with warm water until the contents of the stomach were clear and only water exited the gastric cannula. Rats were then fit with sham feeding tubes and placed in mesh wire floored Plexiglas sham feeding boxes. Rats were acclimated to the sham feeding procedure by overnight (1700 – 0900) water and food deprivation and sham fed 25% corn oil in the morning (0900). The acclimation period was three trials with a day in between each trial. After experimental acclimation, rats underwent the same protocol as acclimation, but were not fasted. Varying concentrations of corn oil (12.5, 25, 50, 75, 100%) were presented in both ascending and descending order of concentration. Intake of the oil concentrations was recorded every 5 minutes for 60 minutes. All drainage from the sham feeding tubes was collected in plastic basins and discarded after each experiment. Sham feeding took place every other day.

Experiment 2: Long-term and Short-Term Food Deprived Sham Feeding

A different group of rats (2-hr deprived: n = 10, 5 OLETF, 5 LETO; overnight deprived: n = 12, 5 OLETF, 7 LETO), were used in overnight food

deprived and 2-hr deprived (0500 – 0900 hrs) sham feeding experiments. Rats were fit with chronic gastric cannulae and allowed at least 2 weeks to recover. At the onset of the experiment, rats were weighed, stomachs lavaged with warm water until contents exiting the stomach were clear of food particles. Sham feeding tubes were then connected to the gastric cannulae and rats placed in Plexiglas sham feeding boxes. To train the rats, we used water deprivation briefly (2 hours) in the morning before sham feeding experiments. Rats were presented with the oil concentrations used in non-deprived sham feeding conditions.

Experiment 3: Real Feeding of oil emulsions

Rats (n = 13, 6 OLETF, 7 LETO) used in the overnight deprived sham feeding tests were used for real feeding experiments. They were allowed ad libitum access to chow until presentation of oils. At 0900 hrs, rats were weighed, placed back into their cages, and given burettes of the same corn oil concentrations as the above protocols. Screw caps sealed the gastric cannulae to assure real feeding.

Experiment 4: Deprived and Non-deprived Oil Preference Tests

Subjects: 34 rats (16 OLETF and 18 LETO), were divided into four groups (2 x 8 OLETF and 2 x 9 LETO) matched for body weight within each strain. Two groups of rats (8 OLETF and 9 LETO) were food deprived the night before tests (1700 – 0800) while the other 2 groups received ad libitum access to rat chow,

except during two-bottle preference testing. For acclimation, rats were presented with either 100% corn oil or 100% mineral oil for a total of 16 days: 4 cycles x 2 days with each oil. Before preference tests, rats' stomachs were gently lavaged with warm water until only water exited the cannula. Sham feeding tubes were then connected and rats placed into Plexiglas sham feeding boxes. Rats received concomitant access to burettes containing 100% corn and 100% mineral oil. To avoid side preference, burette positions were alternated for each test.

Calories Consumed

To examine the amount energy consumed during non-deprived and deprived states, emulsion consumed was multiplied by the percentage of the emulsion and multiplied by nine to obtain the energy consumed in each condition.

Oral Glucose Tolerance Test (OGTT)

Rats (n = 12, 6 per strain) from the non-deprived sham feeding groups were deprived of food overnight and were tested for baseline levels of blood glucose levels in the morning (0900 hours). Glucose (2 g/kg) was given via oral gavage. Tail blood was collected at 0, 30, 60, 90, and 120 minutes post glucose administration. Rats with blood glucose over 300 mg/dl at any time post gavage or over 200 mg/dl at 120 minutes post gavage were considered diabetic. Blood was analyzed using a glucometer (Lifescan, One-Touch Basic).

Statistical Analyses

All statistics were computed using Statistical Analysis Software (SAS, version 9.1.3, Cary, NC). All one-bottle acceptance testing was analyzed by one-way Analysis of Variance (ANOVA) at each concentration for between strain statistical analysis. To reveal within strain differences, we used two-way (strain x oil concentration) repeated measures ANOVA (rmANOVA). Two-bottle preferences tests were analyzed by rmANOVA. OGTT data was analyzed by one-way rmANOVA. For all data, P-values < 0.05 were considered statistically significant.

Results

Non-Deprived Sham Feeding

One-way ANOVA revealed that OLETF consumed significantly more of the 50 and 75% concentrations than LETO (OLETF: 50%: 8.47 ± 1.67 ml, $P < 0.05$; 75%: 8.80 ± 1.86 ml, $P < 0.05$; LETO: 50% 4.79 ± 0.69 ml; 75%: 3.25 ± 0.69 ml), while LETO consumed more oil than OLETF at the 12.5 and 25% concentrations (OLETF: 12.5%: 2.27 ± 0.50 ml, $P < 0.001$; 25%: 3.24 ± 0.48 ml, $P < 0.0001$; LETO: 12.5%: 6.06 ± 0.81 ml; 25%: 8.07 ± 1.05 ml) (Fig. 2.1). Within strains, OLETF rats consumed significantly more of the 50 and 75% oil concentrations compared to 12.5, 25%, and 100% concentrations ($P < 0.01$ for each), while LETO consumed significantly more 25% oil concentration compared to all other concentrations tested ($P < 0.01$).

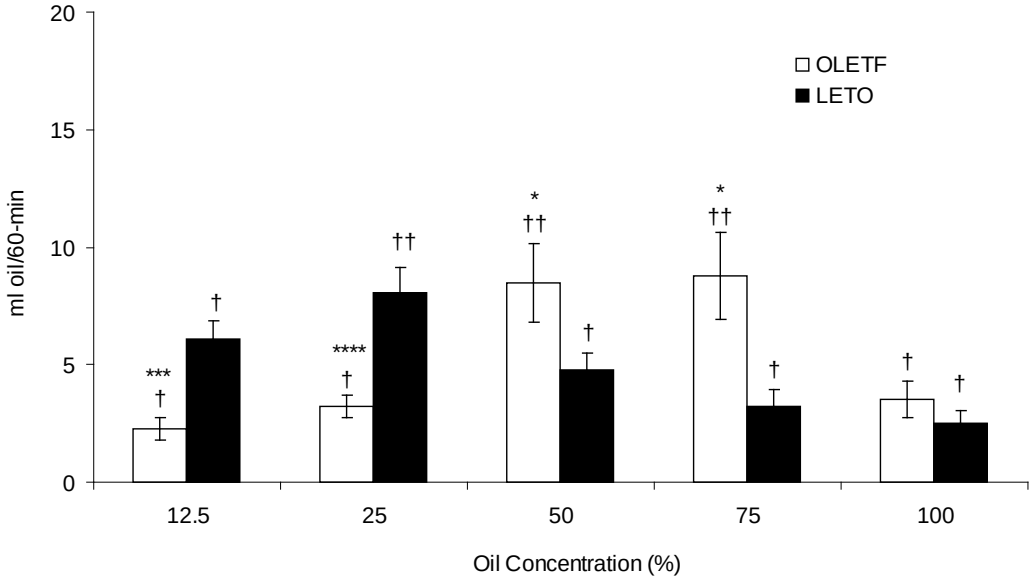

Fig. 2.1: Sham feeding in a non-deprived condition. Between strains, OLETF consumed significantly more 50 and 75% oil concentrations compared to LETO. LETO consumed significantly more 12.5 and 25% oil concentrations compared to OLETF. OLETF consumed significantly more 50 and 75% oil compared to other concentrations and LETO consumed more 25% oil concentration compared to all other concentrations tested. * denotes differences between strains. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$. † denotes the statistical grouping within strains ($P < .01$ within strains)

Overnight Deprived Sham Feeding

Analysis of overnight deprived sham intake between strains, showed that OLETF consumed significantly more 12.5% oil than LETO (OLETF: 12.5%: 64.51 ± 8.30 ml; LETO: 12.5%: 43.81 ± 3.62 ml, $P < 0.05$). Within strains, both OLETF and LETO consumed significantly more 12.5 and 25% concentrations compared to 50, 75, and 100% concentrations ($P < 0.01$ for each) (Fig. 2.2).

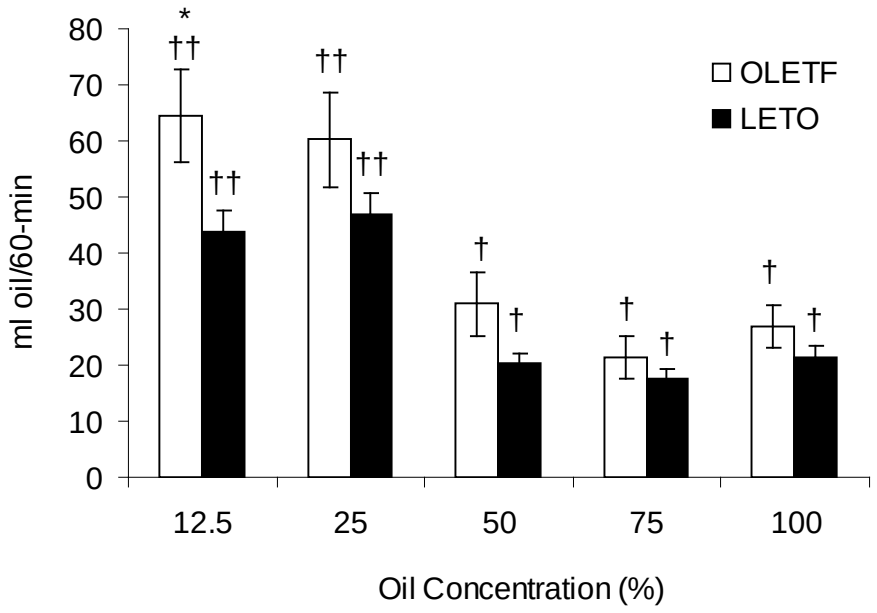

Fig. 2.2: Sham feeding in an overnight deprived condition. OLETF rats consumed significantly more 12.5% oil than LETO. Both OLETF and LETO consumed significantly more 12.5 and 25% oil compared to all concentrations tested. * denotes statistical significance between strains. * $P < 0.05$. † denotes statistical significance within strains ($P < 0.01$).

2-hr Deprived Sham Feeding

After 2-hr deprivation, OLETF rats consumed significantly more oil for the 25 and 50% concentrations compared to LETO (OLETF: 25%: 20.05 ± 2.73 ml, $P < 0.05$; 50%: 17.2 ± and 1.75 ml, $P < 0.01$; LETO: 25% 10.23 ± 2.30 ml; 50%: 8.51 ± 1.34 ml). Within strains, OLETF rats consumed significantly less 100% oil compared to all other concentrations ($P < 0.01$). LETO drank all concentrations in similar volumes (Fig. 2.3).

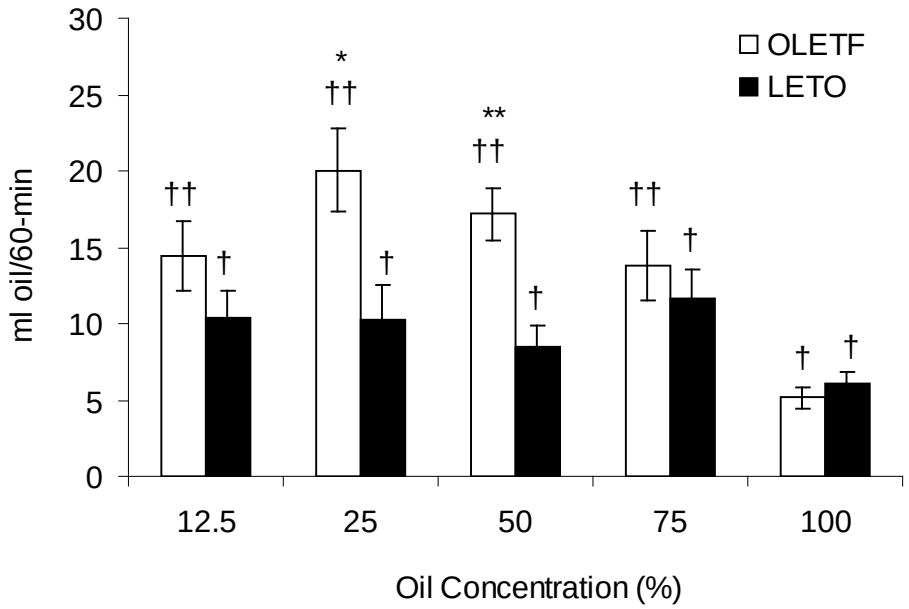

Fig. 2.3: Sham feeding following 2-hr deprivation. Between strains, OLETF consumed significantly more 25 and 50% oil compared to LETO. Within strains, OLETF consumed significantly less 100% oil compared to all concentrations tested. OLETF rats drank significantly less 100% oil emulsion compared to all other concentrations. * denotes statistical significance between strains. * $P < 0.05$, ** $P < 0.01$. † denotes statistical significance within strain ($P<.01$).

Non-Deprived vs. Overnight Deprived Sham Feeding

Statistical analysis of non-deprived sham feeding compared to overnight deprived sham feeding revealed that OLETF consumed significantly more oil at concentrations of 12.5 and 25% after overnight deprivation ($P < 0.0001$ for both). LETO rats consumed significantly more oil at all concentrations after overnight deprivation compared to the non-deprived condition (12.5%: $P < 0.0001$; 25%; $P< 0.0001$; 50%: $P < 0.001$; 75%: $P < 0.01$; 100%: $P < 0.0001$)

Non-Deprived vs. 2-hr Deprived Sham Feeding

Repeated measures ANOVA revealed that OLETF rats consumed significantly more 12.5 and 25% oil after 2-hr deprivation compared to non-deprivation ($P < 0.01$ and $P < 0.0001$, respectively). LETO rats consumed all concentrations similarly in the non-deprived and 2-hr deprived testing ($P = 1.000$ for each).

Overnight Deprived vs. 2-hr Deprived Sham Feeding

Statistical analysis of the overnight deprived and 2-hr deprived sham feeding intakes revealed OLETF rats drank significantly more 12.5 and 25% oil after overnight deprivation ($P < 0.0001$ for each). LETO rats consumed significantly more oil for 12.5, 25, and 100% concentrations after overnight deprivation ($P < 0.0001$ for each).

Real Feeding

One-way ANOVA revealed that OLETF rats consumed significantly more oil at concentrations of 50, 75, and 100% oil compared to LETO (OLETF: 8.41 ± 1.02 ml, $P < 0.05$; 75%: 8.66 ± 0.91 ml, $P < 0.05$; 100%: 5.31 ± 0.57 ml, $P < 0.001$; LETO: 50%: 6.00 ± 0.33 ml; 75%: 5.64 ± 0.64 ml; 100%: 2.73 ± 0.35 ml). Within strain analysis revealed both OLETF and LETO consumed significantly more 12.5 and 25% oil compared to 50, 75, and 100% concentrations (Fig. 2.4).

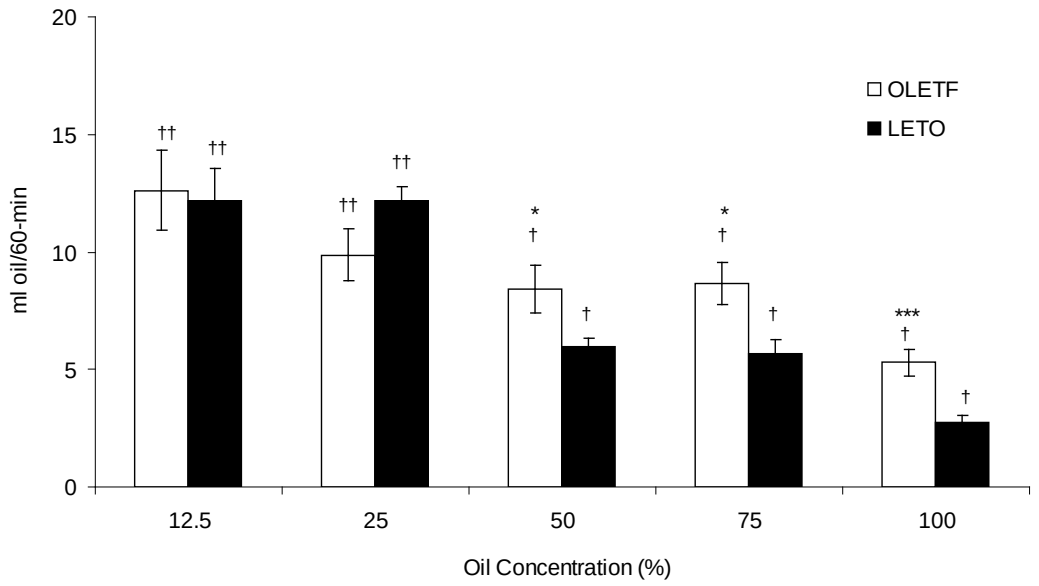

Fig. 2.4: Real feeding in a non-deprived condition. Between strains, OLETF consumed significantly more 50, 75, and 100% oil compared to LETO. Within strains, both OLETF and LETO consumed significantly more 12.5 and 25% oil compared to other concentrations tested. *denotes statistical difference between strains. * $P < 0.05$, *** $P < 0.001$. † denotes statistical significance within strain ($P<.01$).

Calories Consumed

Repeated measures ANOVA revealed in the non-deprived sham feeding tests, OLETF rats consumed significantly more calories from 75% oil than all other concentrations tested ($P < 0.01$ for each). LETO rats, however, consumed similar calories from all oil concentrations tested ($P < 1.000$ for all). In the overnight deprived condition, both OLETF and LETO rats consumed significantly more calories from 100% oil compared to all other oil concentrations tested ($P < 0.01$ for each). After a brief 2-hr deprivation, both OLETF and LETO rats consumed significantly more calories from the 75% oil compared to all other concentrations tested ($P < 0.01$ for each) while LETO rats consumed significantly

more calories from the 75 and 100% oils compared to 12.5, 25, and 50% oils ($P < 0.01$ for each). Finally, in the non-deprived real feeding tests, OLETF rats consumed significantly more calories from the 75% oil compared to all other concentrations tested ($P < 0.01$ for each), whereas LETO rats consumed significantly more calories from the 50, 75, and 100% oils compared to 12.5 and 25% oils ($P < 0.01$).

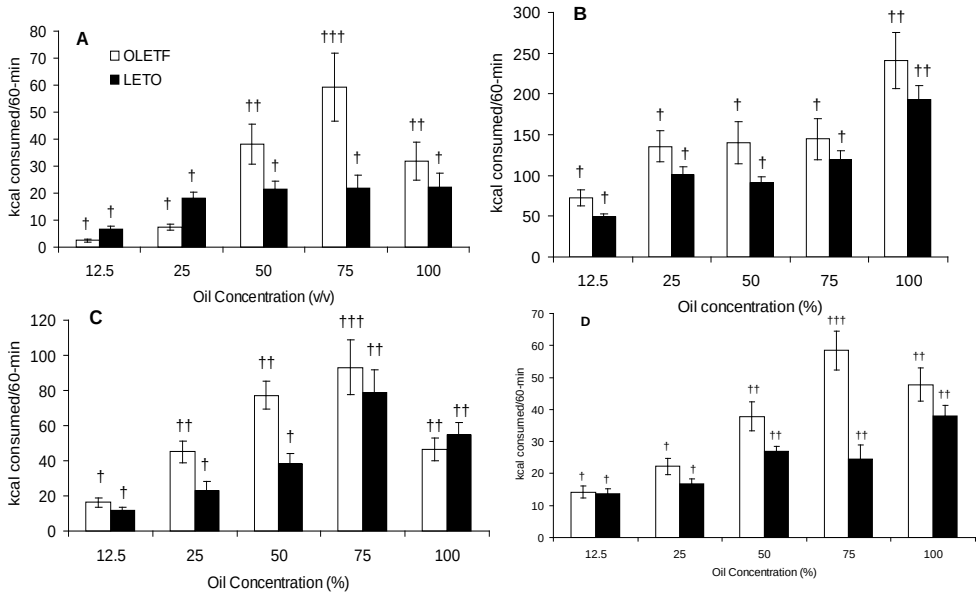

Figure 2.5: Calories consumed in (A) non-deprived sham feeding, (B) overnight deprived sham feeding, (C) 2-hr deprived sham feeding, and (D) non-deprived real feeding tests. † denotes statistical grouping within strain ($P< 0.01$).

Deprived and Non-deprived Oil Preference

In the non-deprived condition two-bottle preference test results, both LETO and OLETF preferred corn oil over mineral oil ($P <.01$). During deprived two-bottle testing, OLETF preferred corn oil to mineral oil ($P < .01$), while the

LETO rats preferred both corn oil and mineral oil equally. Between strains in the deprived condition, OLETF rats preferred corn oil significantly more than the LETO rats ($P < .01$). A comparison for each strain across conditions yielded no significance difference in preference for either corn oil or mineral oil (Fig. 2.5).

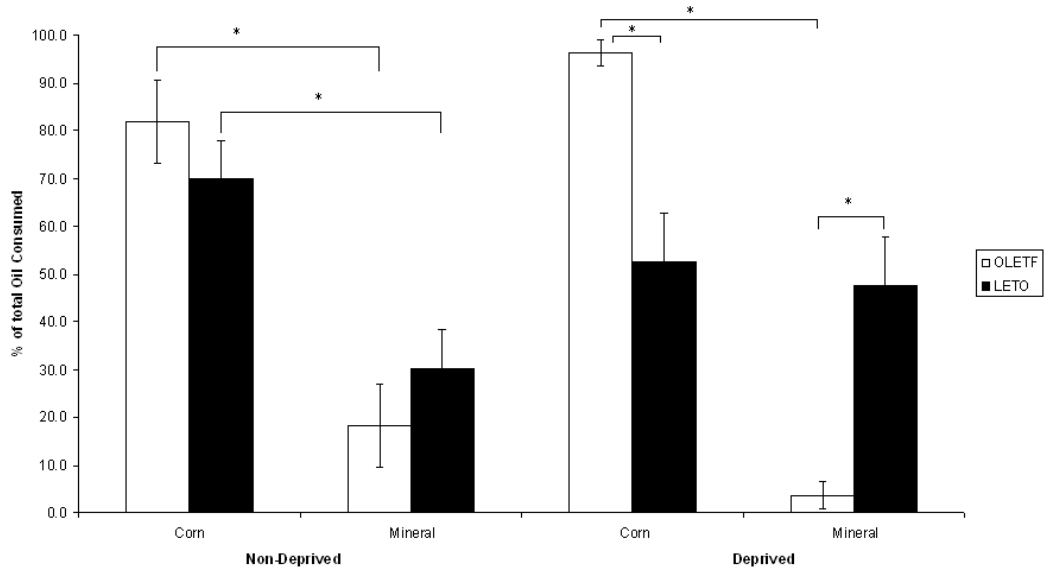

Fig. 2.6: Preference tests in non-deprived and deprived OLETF and LETO rats. Between strains, OLETF preferred corn oil significantly more than LETO. After overnight deprivation LETO preferred mineral oil more than OLETF after overnight deprivation. OLETF and LETO both preferred corn oil to mineral oil while non-deprived. After overnight deprivation, OLETF preferred corn oil to mineral oil and LETO preferred both oils equally. * denotes $P < .01$.

Oral Glucose Tolerance Test (OGTT)

Resting blood glucose levels in OLETF and LETO rats were not significantly different at any time-point post gavage (Fig. 2.6). (0 min: $P = 1.000$; 30 min: $P = 0.176$; 60 min: $P = 0.318$; 90 min: $P = 1.000$; 120 min: $P = 1.000$). OLETF rats were still considered pre-diabetic by blood glucose testing standards.

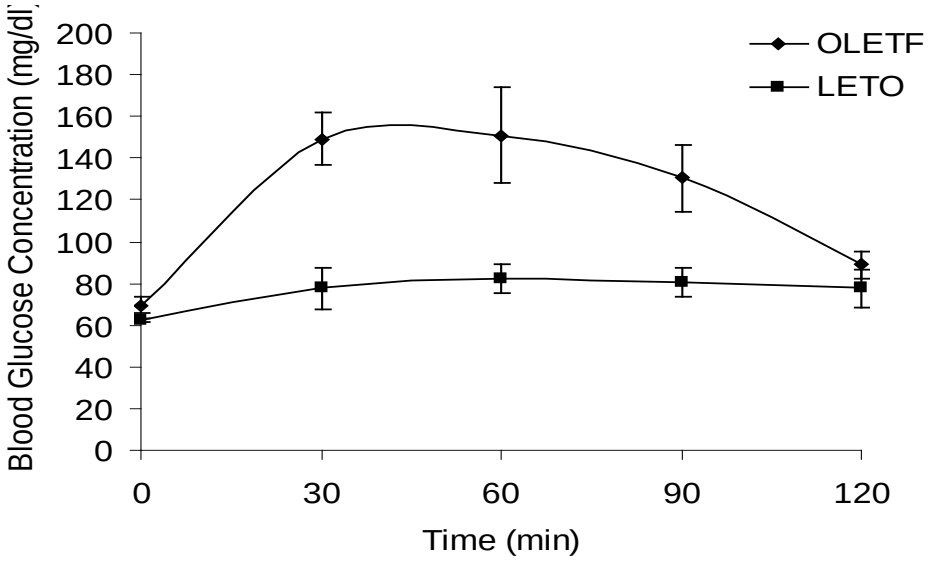

Figure 2.7: Oral glucose tolerance test. OLETF rats maintained elevated blood glucose levels from 30 to 90 minutes post glucose gavage.

Discussion

Our study shows that prediabetic OLETF rats sham fed increased amounts of higher concentration of oils compared to LETO controls in a fed state; however, when deprived, the concentration curve shifted to the left towards lower concentrations. Additionally, our results show that in the presence of gastrointestinal feedback, OLETF rats fed significantly increased amount of the high oil concentrations compared to LETO. Finally, we have shown that OLETF preferred corn oil to mineral oil during both deprivation and non-deprivation conditions. In contrast, LETO preferred corn oil when food deprived, while they consumed equal amounts of corn and mineral oil when non-deprived. These

results show for the first time that OLETF rats have an increased avidity to oils and consume more oil at a high concentration compared to LETO rats.

Previous work from Mindell et. al (69) showed that oil intake in one-bottle acceptance test, is dependent upon concentration, with the highest intakes occurring at 12.5 to 50% oil concentration. In our study, in the non-deprived condition, OLETF rats' oil intake was higher at the 50 and 75% concentrations compared to LETO. LETO, however, drank higher amounts of 12.5 and 25% oil compared to OLETF rats. In other words, OLETF's oil consumption while non-deprived was shifted towards the right of the concentration curve compared to LETO rats. After a brief deprivation, however, OLETF oil consumption, relative to LETO, was significantly altered resulting in a leftward shift in the concentration curve.

Oil intake is stimulated through orosensory mechanisms and inhibited intake through gastrointestinal feedback, though gut feedback also has been shown to have a stimulatory effect. Prior work in the OLETF rat has examined deficits to peripheral satiation signals, which were largely attributed to the lack of a functional CCK-1R (70). Our sham feeding data isolated the orosensory component of oils, while limiting negative feedback from the gastrointestinal tract. Therefore, we were better able to assess the contribution of stimulatory component of oils in an attempt to dissect oil consumption and preference to varying concentrations of oils.

To determine whether changes in oil consumption based on orosensory characteristics are driven by overall caloric needs, we employed a food

deprivation schedule. Our results show that when deprived, OLETF rats will significantly alter their pattern of oil intake compared to a non-deprived state. When deprived overnight, OLETF rats not only drank significantly more of the low (12.5%) oil concentration than LETO, but also drank significantly more 12.5 and 25% oil compared to higher concentrations. After a brief, 2 hour deprivation period, OLETF rats drank significantly more oil at 25 and 50% concentrations compared to LETO. However, within strain comparison showed all but the 100% concentration were equally consumed by OLETF rats. Therefore, compared to LETO, OLETF rats increased their caloric intake to compensate for the energy deficits. It has been shown that OLETF rats have altered homeostatic energy regulation, and overcompensate when food deprived (7). The fact that both low and high concentrations of oils were increased after deprivation, suggests that OLETF rats' increased oil intake was driven by energy deficit as well as gustatory functions.

Our results obtained in one-bottle sham feeding tests in deprived conditions are particularly interesting when compared to previous work performed in the Zucker fatty rat. After overnight deprivation, sham fed Zucker fatty rats drank increased amounts of 50 and 75% oils compared to 12.5, 25, and 100% oil concentrations; however, their lean controls also exhibited the same pattern in oil intake (42, 103). Our results, differ from this in that LETO rats exhibited one-bottle intakes similar to that of Sprague-Dawley rats (maximal intake between 12.5 and 50%) while OLETF rats consumed significantly high amounts of 50 and 75% oils compared to LETO. Hence, while Zucker rats'

hyperphagia on a HF diet (13) and preferred fatty foods (16) may be due to decreased gastrointestinal feedback, the OLETF rats' hyperphagia of fatty foods (94) are due both to the increased orosensory stimulation observed in our study, as well as decreased inhibitory feedback from peripheral satiation signals previously established.

To further examine the intricacy between the oral and gastrointestinal component of oils, we examined real feeding in deprived OLETF and LETO rats. From our results, we found that OLETF and LETO rats consumed similar amounts of low (12.5 and 25%) oil concentrations; however at moderate (50%) and high (75 and 100%) oil concentrations, OLETF rats significantly increased oil consumption relative to LETO rats. Previous data from Dr. Covasa (21), as well as Schwartz et. al (94) show OLETF rats were less sensitive to the satiating effects of lipids, which is due to peripheral satiation deficits. These results show that both the OLETF rats' increased oral sensitivity, as well as peripheral satiation deficits contribute to an increased consumption of highly fat solutions.

In an attempt to examine preferences of non-nutritive and nutritive oils, we sham fed corn oil and mineral oil in two-bottle preference tests. From these results, we found that OLETF rats prefer corn oil whether deprived or non-deprived; however, LETO preferred corn oil to mineral oil when non-deprived and preferred both oils equally when deprived. Normally, rats prefer corn oil to mineral during two-bottle preference test in both deprived and non-deprived conditions (69). While OLETF rats exhibited this trend, LETO rats demonstrated the contrary in the non-deprived condition. One caveat, however, to this result in

the LETO is the large variability in preference from the first test day to the second. While on the first day, a majority of LETO rats preferred corn oil over 90% to mineral oil, the second day, a select group of rats, which had previously preferred corn oil almost exclusively, preferred mineral oil to corn oil. Although this result appears to be due to acquisition of side preference, we had attempted to curtail this by requiring all rats to sample the first oil presented prior to presenting the second oil.

While the mechanisms underlying the stimulation of oil intake are largely unknown, some current evidence may provide light on the topic. Previously, olfaction was thought to control discrimination of oils; however, rats are able to detect solid fats mixed with chow even after rats are anosmic (69). Though tacticle mechanisms are correlated to fattiness of foods in humans (68), scant data exists in rodent models (50). Taste appears to be another pathway as well in controlling oil intake in the absence of gastrointestinal feedback. The fatty acid transporter, CD36, is located on taste cells (36, 54) and correlates with increased preference of lipids (99); however, this evidence is not yet concrete. Additional research shows fatty acids can depolarize taste cells (39). Further, lingual lipase, which is present in rodents and infants, hydrolyzes triglycerides in the oral cavity (51). Although this may be interesting, the supporting data are far from complete to implicate it as a controlling mechanism of oil intake. Recent work also points towards the central processing of oils. Dopamine release occurs after the sham feeding of either sucrose (49) or oil (62) and further, dopamine antagonists efficiently inhibit oil intake in sham feeding rats (114). Finally, work

from Hajnal et al. (47) shows that OLETF are more susceptible than LETO rats to the inhibitory effects of dopamine antagonists on sham feeding of sucrose. From these data, several possibilities exist to explore the increased sensitivity to oil in OLETF rats.

Although we do not yet clearly understand the controls of oral stimulation in oil intake, we have shown that OLETF rats increased acceptance and thus, consumption of high concentration oils compared to LETO rats. However, even a brief deprivation affects this acceptance in a left-shifted manner, causing OLETF to consume higher amounts of low concentration oils. In the presence of both orosensory stimulation and gastrointestinal feedback, we found that OLETF rats maintain their increased consumption of high concentration oils, most likely due to both peripheral and central reward mechanisms

Chapter 3

CCK SENSITIVITY AND FOS-LI IN DIO AND DR RATS

Introduction

Obesity is a global epidemic. In the United States, an estimated 66% of the population is overweight, of the overweight population, approximately 50% are obese. Because the prevalence of obesity has increased dramatically over the last 20 years, it is very unlikely that genetic factors are responsible for this epidemic (3). Rather, dietary changes most likely are the cause for the majority of obesity. Consumption of a HF diet for a lengthened period of time is correlated with increased energy intake, and thus, obesity (116). Additionally, studies in humans have shown that some individuals, when consuming a HF diet, may be resistant to developing obesity while others are more susceptible (for review see (10)). One mechanism that may be responsible for overconsumption and subsequent obesity in some individuals is the reduction in sensitivity to satiation signals, which serve to terminate a meal (22, 72, 106, 113). Therefore, in order to study the controls of food intake while exposed to a high fat diet, diet induced obese (DIO) and diet induced obese resistant (DR) models have been developed due to their phenotypes being inherited as polygenic traits, which most resembles human obesity (12, 107)

When subjected to a HF diet, DIO rats will increase overall energy intake by means of increasing meal size, rather than meal frequency (31). Additionally,

when subjected to a HF diet, first generation DIO rats increase body weight substantially compared to DR rats, which is accompanied by significantly increased adiposity as well as insulin resistance. Furthermore, established lines of DIO rats will gain body weight at a higher rate than DR rats while on a standard rodent chow diet, but maintain insulin sensitivity (56). While little investigation has been performed to examine potential peripheral satiation signal deficits in DIO rats, a growing amount of literature has shown consumption of a high fat diet decreases sensitivity to the satiation signal CCK (22, 90).

Released from the I-cells of the duodenum in response to fatty acids and hydrolyzed proteins, CCK serves a signal to terminate meals by affecting pancreatic secretion, gastric emptying, and gut motility. Previous studies from our laboratory have shown that when rats were maintained on a HF diet, the satiation effects of CCK were significantly decreased compared to low fat fed rats (90). In obese models, CCK limits food intake; however, work with Zucker fatty (fa/fa) rats found resistance to exogenously administered CCK, thus food intake was unchanged (75). Because resistance to CCK begins at a young age in obese models, the reduction in sensitivity to satiation signals may be responsible for the subsequent hyperphagia and obesity. The fact that DIO rats increase meal size and not meal frequency when placed on a HF diet, may suggest a potential deficit to the satiating effects of CCK. So far, only one study examined responses to exogenous CCK in DIO rats. In this study, Chandler et al., showed that after administration of a single dose of CCK (0.3 μg/kg) previously found to be subthreshold in outbred Sprague-Dawley rats, DIO rats suppressed food

intake significantly more than DR rats (17). Furthermore, while DIO rats gain more weight on a chow diet compared DR rats; it is not known whether this is due to a decreased sensitivity to peripheral satiation signals, such as CCK. In order to systematically examine the differential responses of sensitivity to CCK between DIO and DR rats, the present study assessed food intake responses following various doses of CCK (2.0, 4.0, and 8.0 μg/kg) when rats were maintained on a regular chow diet. To elicit satiation, CCK binds to CCK-1R located on vagal afferent fibers (104), which are located distally in the intestinal mucosa. Additionally, CCK exposure results in a vagal-dependent increase of Fos-like immunoreactivity (Fos-Li) in the NTS of the dorsal hindbrain (23), where the proximal terminals of vagal afferents synapse. While, Fos-Li is a marker of neuronal activation, it is also strongly correlated with food intake in the NTS (119). To examine whether feeding behavioral responses correlate with changes in vagal signaling to the hindbrain we examined Fos-Li immunoreactivity in DIO and DR rats following CCK administration.

Finally, to assess differences in weight gain between DIO and DR rats on a chow fed diet, we measured 24-hr food intake and dissected fat pad depots(epididymal, retroperitoneal, visceral) to examine if DIO have a greater adiposity than DR rats. Diabetic status was assessed using an oral glucose tolerance test (OGTT).

Methods

Animals

A total of twenty-four (n = 24) 5-week old DIO and DR rats were obtained from Charles River Laboratories (Wilmington, MA) and used in all experiments. All animals were housed in steel hanging wire cages in a temperature controlled vivarium with a 12:12 hr light/dark cycle (lights on at 0600 h). Throughout experiments, rats were fed standard rat chow (Purina 5001). All animals were acclimated to their environment and handled for at least one week before experimental protocol began. All experimental protocols were approved by the Pennsylvania State University's Institutional Animal Care and Use Committee (IACUC).

24-hr Food Intake

After CCK sensitivity tests, 24-h food intake was measured for 5 days in all rats. Pre-weighed rat chow pellets were placed in the cages at 1700h with aluminum trays under all cages to account for spillage. Food was removed at 1700 h the next day and was replaced with pre-weighed chow.

Fat Pad Analysis

Animals were deeply anesthetized via an intramuscular injection with a Xylazine/Ketamine/AcePromazine (1 ml/kg) cocktail and perfused intracardially with 0.1 M phosphate buffer solution followed by 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). After perfusion, visceral, epididymal, and

retroperitoneal fat pads were excised and collected. Fat pads were weighed to the hundredth of a gram. Due to body weight differences between strains, fat pads were also expressed as a percentage of body weight (fat pad weight (g)/ bodyweight (g)*100.

Oral Glucose Tolerance Test (OGTT)

Half of the rats (n = 6 DIO, n = 6 DR) were used to assess glucose tolerance across strains. Rats were deprived overnight (1700 – 0900h) and orally gavaged with a 50% glucose solution (w/v) at 2 g glucose/kg bodyweight using 8 French tubing. Baseline blood glucose levels were obtained pre-gavage and blood glucose levels were measured at 30, 60, 90, and 120 minutes post gavage. All blood glucose values were obtained via glucometer (One Touch Ultra).

CCK Sensitivity

The day before each trial (1700), chow was removed from half the rats each day (n = 6 DIO, n = 6 DR). Approximately at 0900 h on the next morning, food deprived rats were weighed and subjected to intraperitoneal (IP) injections of cholecystokinin octapeptide (CCK-8) or saline vehicle. Before experimental protocol occurred, rats were acclimated to (IP) injections with a saline vehicle for at least 3 trials, until a stable baseline was achieved. Doses of 2, 4, and 8 µg/kg were used in random order to establish CCK sensitivity between strains. After 5-minutes post injection of CCK or saline vehicle, rats were given solid-pelleted rat

chow and intake was measured at 30 and 60 minutes. Aluminum trays were placed under each cage to account for any spillage incurred throughout the experiment. After each experiment, rat chow was returned to the cages. Groups were alternated every other day so that one group always received either CCK or saline.

Fos-Like Immunoreactivity (Fos-Li)

To examine possible differences in CCK-induced Fos-li between strains, rats used in previous experiments were divided into three separate groups. Fos-Li was then examined in the dorsal hindbrain of rats after IP administration of the following treatments: saline, 4.0 or 8.0 μg/kg CCK. The experimental protocol and the doses used were identical to the ones used in the behavioral experiment described above examining the effects CCK sensitivity, except that the overnight fasted rats were not given access to pelleted rat chow. Ninety minutes after treatments, rats were anesthetized and perfused as described above. After tissue fixation, brains were removed, stored for 4h in 4% paraformaldehyde and then transferred to 20% sucrose solution for overnight storage. Free-floating brain sections in Tris-buffered saline were cut in 40-μm coronal slices using a cryostat followed by 50% ethanol solution wash for 30 min. Sections were incubated in 10% normal horse serum followed by a 24-h incubation in rabbit polyclonal antiserum raised against a peptide corresponding to residues 2–17 of human and mouse Fos-Li (Ab-5) at a dilution of 1:50,000. Next, sections were

incubated in a 1:500 dilution of a biotinylated antibody raised in donkey, against rabbit IgG (Jackson Immuno-Research Laboratories, Inc., West Grove, PA) for 18 h, followed by a 3-h incubation in avidin conjugated to horseradish peroxidase (Sigma, St. Louis, MO). The avidin–biotin–antibody complex was revealed histochemically using nickel-intensified diaminobenzidine reaction. After being mounted on slides, cleared and cover-slipped, brain sections were inspected microscopically and counts of Fos-Li nuclei were made. The counts for each section were done manually by two individuals who were blinded to the treatments at four different brain levels corresponding to −13.30 mm, −13.80 mm and −14.08 mm behind bregma, according to the stereotaxic atlas of Paxinos and Watson. Fos-Li neurons in the following brain structures were counted bilaterally for each rat in each coronal section corresponding to one of the four rostro-caudal levels comprising the NTS, AP and the dorsal motor nucleus of the vagus (DMV). A minimum of six sections per each brain level were analyzed for each treatment. The presented data are the counts of Fos-Li for a given nuclei averaged within and across each of the rostrocaudal levels.

Statistical Analyses

All statistics were computed using Statistical Analysis Software (SAS, version 9.1.3, Cary, NC). Twenty-four hour food intake was analyzed through one-way (strain) repeated measures Analysis of Variance (rmANOVA). Weekly body weights, gross fat pad weight, and relative fat pad mass based on body weight were analyzed by two-way unpaired student t-test with Bonferroni

adjustment. Blood glucose levels were analyzed by one-way (strain) rmANOVA with Bonferroni adjustment. Suppression of food intake by CCK-8 was analyzed by two-way (strain x treatment) rmANOVA with Bonferroni adjustment. Fos-Li counts were analyzed with one-way (strain) ANOVA for each treatment. All data are expressed as means ± SEM. Significance was determined by a P-value < 0.05.

Results

24-Hour Food Intake, Body weight, and Fat Pads

Repeated measures ANOVA revealed that 24-hr food intake in DIO rats was significantly higher compared to DR rats (DIO: 22.3 ± 0.81g; DR: 19.56 ± 0.77g; $P < 0.05$) (Fig. 3.1).

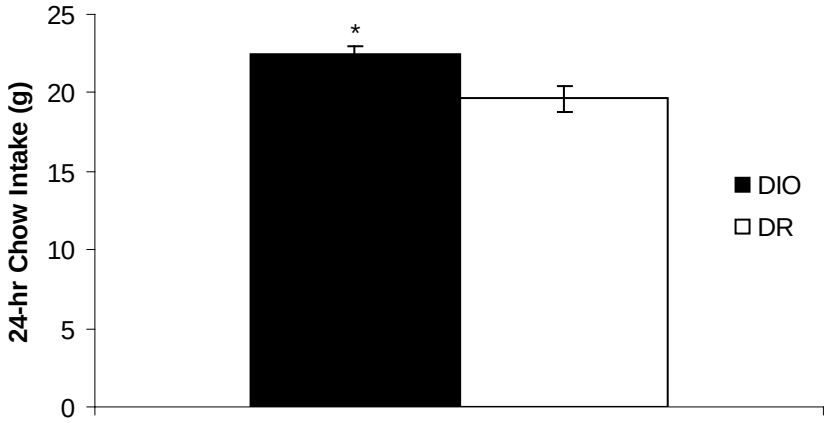

Fig. 3.1: Daily amount of chow consumed (grams ± SE) during a 5 day period. The DIO rats consumed significantly more chow than the DR rats during the 24 hour intake period. * denotes P<0.05, compared to DR.

Student's t-test revealed bodyweights of DIO rats were significantly elevated compared to DR rats beginning at week six and this trend persisted throughout the experiment until rats were sacrificed at week 12 ($P < 0.0001$) (Fig. 3.2).

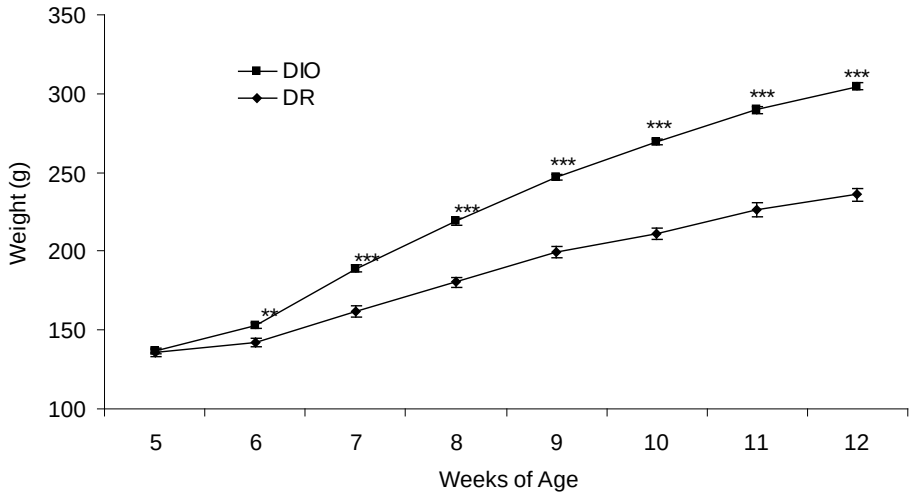

Fig 3.2: Average body weight (± SE) of DIO and DR rats during experiments. DIO rats had significantly higher average body weight than the DR rats. ** denotes P<0.001, *** P<0.0001, between strains.

Overall, the total fat pad weight was significantly higher in DIO than DR rats. (Table 3.1). However, of the three fat pads, only epididymal fat pads weighed significantly more in DIO compared DR rats. The visceral and retroperitoneal fat pad weights although were slightly heavier in DIO compared to DR rats, they did not reach statistical significance. When the fat pads weights were calculated as percentage of bodyweight, DIO rats had less relative fat pad mass than DR rats (3.78 ± 0.07 vs. 4.30 ± 0.22 %). The epididymal fat pad weight was significantly lower in the DIO vs. DR rats while there were no difference in the weights of

retroperitoneal (P = 0.078) and visceral (P = 0.103) fats pads between the strains.

Fat Pad	DIO	DR	Relative Fat Pad Mass (g fat/g BW * 100)	
			DIO	DR
Epididymal (g)	4.50 ± 0.17*	3.84 ± 0.14	1.15*	1.28
Retroperitoneal (g)	5.53 ± 0.19	5.02 ± 0.39	1.41	1.67
Visceral (g)	4.76 ± 0.18*	4.00 ± 0.16	1.22	1.33
Total (g)	14.8 ± 0.31*	12.90 ± 0.63	3.78*	4.30

Table 3.1: Fat pad weight in grams (± SE) of DIO and DR rats. DIO had significantly heavier epididymal, visceral and total fat pad depots compared to DR rats (P<0.05). However, DIO rats had significantly lower epididymal and total relative fat pad mass as percentage of bodyweight (P<0.05). * denotes P<0.05, compared to DR rat fat pad.

CCK Sensitivity

Repeated measures ANOVA revealed main effects of treatment ([5,624] F = 32.81, P <0.0001) and strain x treatment interaction [5, 624], F = 2.84, P < 0.05, but not strain (P = 0.188). For the 2 µg/kg dose, between strains, DIO and DR were not statistically significant as a measure of percent suppression (DIO: 17.20 ± 4.15% vs. DR: 13.66 ± 2.27%, P = 0.452) (Fig. 3.3). Within strains, DIO and DR rats significantly decreased 60-min chow intake (DIO: 2 µg/kg: 7.20 ± 0.31g vs. saline 8.75 ± 0.24g; P < 0.0001; DR: 2 µg/kg: 8.27 ± 0.32g vs. saline 9.55 ± 0.24g; P < 0.0001). After the 4 µg/kg treatment, between strains, DIO suppressed intake significantly more than DR (DIO: 21.07 ± 2.99% vs. DR: 12.65 ± 2.54%, P < 0.05). Within strains, DIO significantly decreased intake compared

to saline (4 µg/kg /kg: 6.85 ± 0.43 g, vs. saline: 9.15 ± 0.28 g. P < 0.0001); however DR did not significantly decrease intake compared to saline (4 µg/kg: 6.85 ± 0.43 g, vs. saline: 9.15 ± 0.28 g). For the 8 µg/kg dose, percent suppression was not statistically significant between strains (DIO: 16.08 ± 4.32% vs. DR: 27.44 ± 5.63%, P = 0.130). Within strain, DIO and DR both significantly decreased intake compared to saline baseline (DIO: 8 µg/kg: 7.87 ± 0.53g vs. saline 9.52 ± 0.31g; P < 0.05; DR: 8 µg/kg: 7.20 ± 0.54 g vs. saline: 9.82 ± 0.29 g, P < 0.0001).

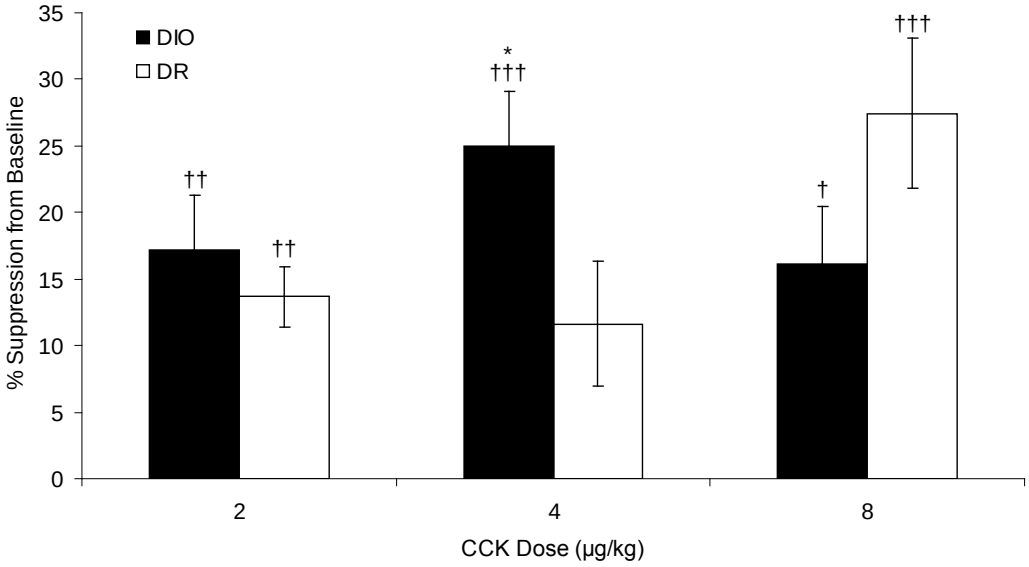

Fig. 3.3: Suppression of chow intake (% from baseline ± SE), following an injection (IP) of CCK-8 at doses of 2.0, 4.0, and 8.0 µg/kg. DIO rats significantly decreased food intake at all doses of CCK tested, while DR rats significantly suppressed from saline at 2.0 and 8.0 µg/kg doses. * denotes statistical difference between strains, † denotes statistical significance from respective saline baseline. † P < 0.05, †† P < 0.001, ††† P < 0.0001.

In both strains, there was a high individual variability in grams of chow intake consumed. To examine this, we plotted the average intake for individual DIO and DR rats at each dose of CCK-8 tested. After examining individual variability in response to CCK-8, we found that the grams of chow consumed by DIO had a more narrow range than DR at each dose tested (Fig. 3.4) (DIO: 2 µg/kg: 4.3 g; 4 µg/kg: 5.1 g; 8 µg/kg: 3.5 g; DR: 2 µg/kg: 5.7 g; 4 µg/kg: 8.2 g; 8 µg/kg: 7.9 g).

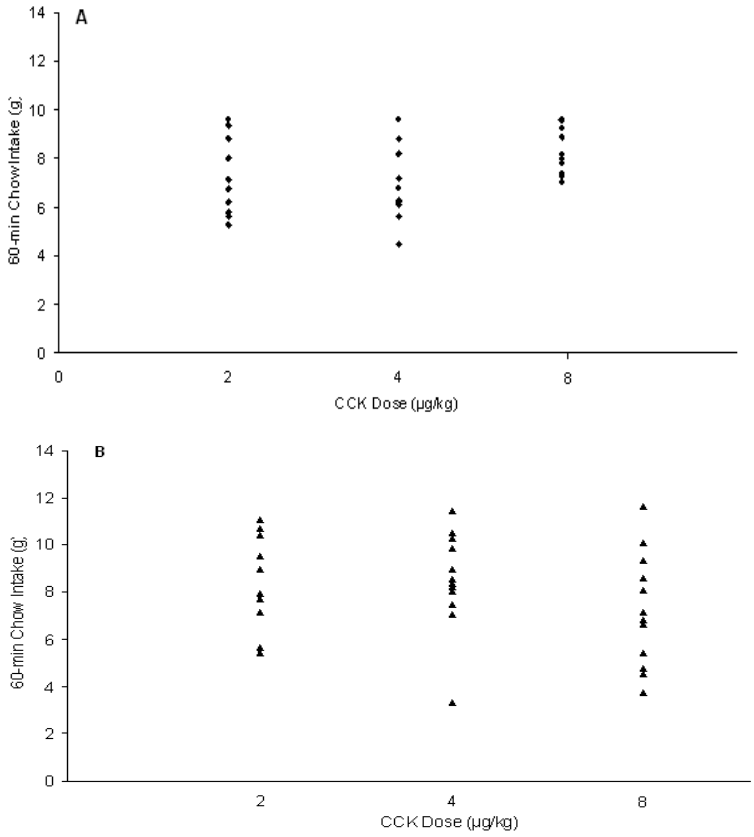

Fig. 3.4: Individual variability plots of (A) DIO and (B) DR rats to CCK-8 dosages of 2.0 µg/kg, 4.0 µg/kg, and 8.0 µg/kg. Data is expressed in grams of chow intake for 60 minutes.

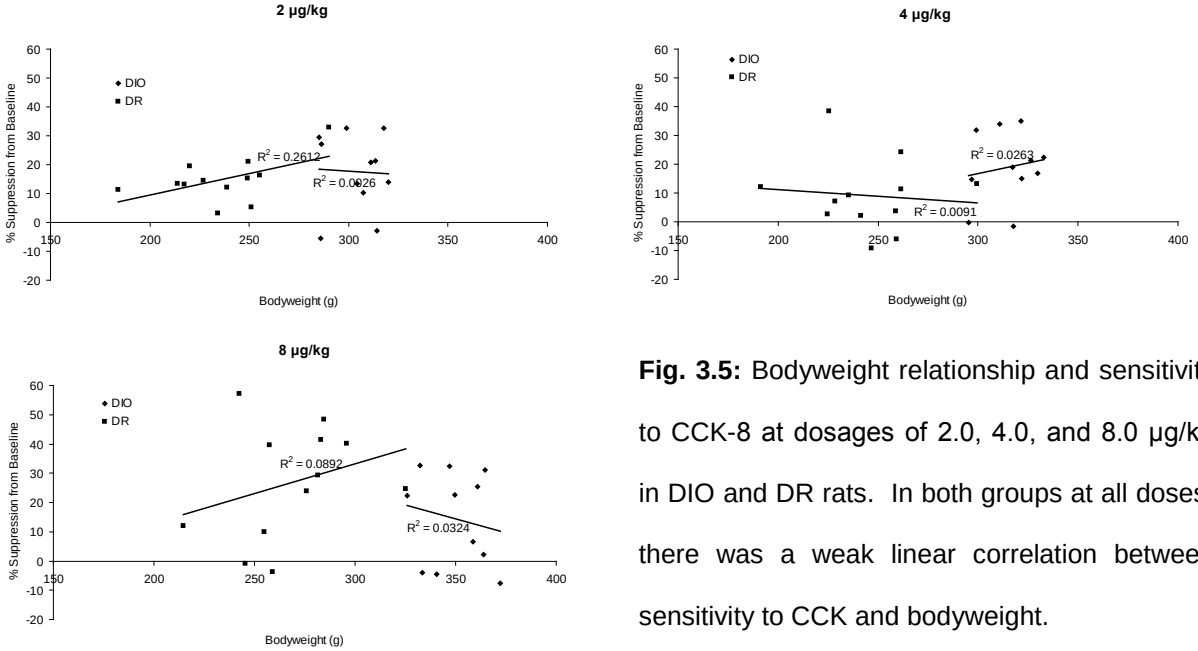

Fig. 3.5: Bodyweight relationship and sensitivity to CCK-8 at dosages of 2.0, 4.0, and 8.0 µg/kg in DIO and DR rats. In both groups at all doses, there was a weak linear correlation between sensitivity to CCK and bodyweight.

Oral Glucose Tolerance Test

Two-way repeated measures ANOVA showed a significant main effects of strain ($F[1,10] = 9.99$, $P < 0.05$), time ($F[4,40] = 162.94$, $P < 0.0001$) and strain x time interaction ($F[4,40] = 7.06$, $P < 0.001$) in the glucose tolerance test. Both DIO and DR rats' blood glucose levels were significantly increased from baseline from 30 through 120 minutes post gavage (Fig. 3.6) ($P < 0.0001$ for all time points). Baseline blood glucose levels in DIO and DR rats were not significantly different (DIO: 51.3 ± 3.6 mg/dl; DR: 50.2 ± 3.6 mg/dl, $P = 1.00$). However, 30 and 60 minutes post gavage, DIO rats' blood glucose levels were significantly decreased compared to DR rats (DIO: 30 min: 109.3 ± 3.6, $P <0.01$; 60 min: 100.7 ± 3.6 mg/dl, $P < 0.01$; DR: 30 min: 131.7 ± 3.6 mg/dl; 60 min: 123.3 ± 3.6 mg/dl). At 90 and 120 minutes post gavage, blood glucose levels across strains were not significantly different (DIO: 90 min: 94.0 ± 3.6 mg/dl; 120 min: 85.8 ± 3.6

mg/dl; DR: 90 min: 123.3 ± 3.6 mg/dl, P = 1.000; 120 min: 87.2 ± 3.6 mg/dl, P = 1.000).

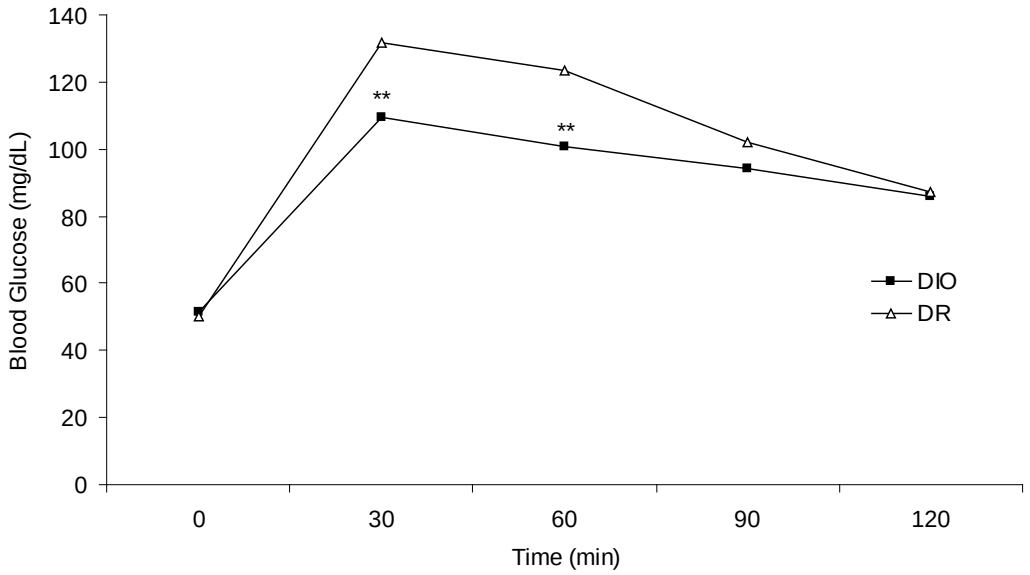

Fig. 3.6: Average blood glucose levels (mg/dl ± SE) in DR and DIO rats. At 30 and 60 minutes post gavage (2 g/kg glucose) DIO rats blood glucose was significantly lower than in DR rats (P<0.01). ** denotes P<0.01, compared to average DIO rat blood glucose.

CCK-induced Fos-Like immunoreactivity (Fos-Li)

After the 4 µg/kg CCK treatment, DIO rats expressed significantly more Fos-Li than DR rats in the NTS (-13.30 mm: P < 0.05; -13.80 mm: P < 0.0001; -14.08 mm: P < 0.01); however, in the DMV, there were no significant differences between strains (P = 1.000 for all levels) (Fig. 3.7). In the AP, there was a significant increase in Fos-Li in DIO compared to DR rats at the level of -13.80 mm (P < 0.05), but not -14.08 mm (P < 0.982). In the 8 µg/kg treated rats, there were no differences in Fos-Li expression between strains. Both doses of CCK in

each strain resulted in significantly increased Fos-Li at all levels from saline treated animals.

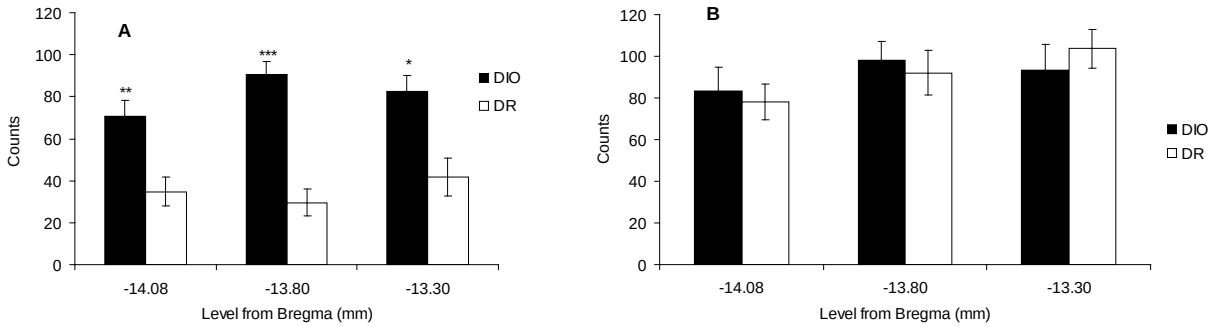

Figure 3.7: Fos-Li counts in the NTS in DIO and DR rats after treatment of (A) 4.0 and (B) 8.0 µg/kg CCK-8. Both strains had significantly increased Fos-Li in the NTS at all brain levels compared to saline treatment. DIO rats had significantly increased levels of Fos-Li compared to DR rats in the NTS at all brain levels after treatment with 4.0 µg/kg. * denotes $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$. There were no difference in Fos immunopositive nuclei between DIO and DR rats following the 8.0 µg/kg CCK-8.

Discussion

The results from this study showed that DIO rats are slightly hyperphagic on a regular chow diet compared to DR rats over a 24-hr period. Subsequently, DIO rats gained weight at a greater rate than DR rats, with significantly increased body weight starting at six weeks of age. While DIO rats were heavier than DR rats, they also had increased raw fat pad depot weight; however, when normalized as a function of bodyweight, DIO rats had less total relative fat pad mass than DR rats. Additionally, fasting blood glucose levels in DIO and DR rats were similar; however, DIO were more glucose tolerant than DR rats as shown

from the OGTT test. We also found that compared to DR, DIO rats responded differently to the satiating effects of CCK. Specifically, we found that DIO rats suppressed food intake significantly more following 4.0 μg/kg dose of CCK compared to DR; however after 8.0 μg/kg CCK-8, DIO were less sensitive than DR rats, but this difference was not statistically significant. When CCK sensitivity was plotted as a function of bodyweight, we found only a small but no significant correlation in both DIO and DR rats. Finally, DIO rats expressed significantly increased amounts of fos-positive nuclei in the NTS at all levels examined in the hindbrain in response to 4.0 μg/kg dose of CCK. However, after treatment with 8.0 μg/kg there was no difference in Fos expression between DIO and DR rats.

Previously, Levin et al. (56) has shown that inbred DIO rats fed chow were approximately 30% heavier than DR while maintaining similar food intake over 24 hours. In our study, we found that DIO rats were 29% heavier than DR by week 12, which is nearly identical with previous data; however, our results differ in that the DIO rats significantly increased food intake over 24 hours. The differences in 24 h food intake between Levin's and our study may be due to the fact that our rats were trained to an overnight food deprivation regimen every 48h. Although we measured 24 h food intake after a two week recovery period from previous tests, it is still likely that the DIO rats were consuming more food to compensate for earlier food deprivation periods (11). Also, we only measured 24 h food intake over a 5 day period while a longer period was assessed in previous work (56). Further, examination of Levin's study revealed that inbred DIO had 44% more carcass fat than DR (56). In this study, although we did not look at overall

carcass fat, the relative fat pad mass, when normalized for bodyweight, was significantly decreased in DIO rats compared to DR. Since we only examined retroperitoneal, epididymal and inguinal fat pads, it is possible that the remaining fat, including subcutaneous, may account for the differences seen in previous studies. However, it is unlikely that this body fat was responsible for the 40% increase that was reported in the DIO rats. Further, work done with mice has shown gonadal fat pad to be highly correlated with body fat percentage (87). As stated above, the food deprivation regimen during the experimental period may had contributed to lower body fat in DIO rats which would explain an increase in food intake. Finally, DIO inbred rats' plasma blood glucose levels have been found to be similar to DR in the fasting state (56). While our results are consistent with this finding, we have further shown that DIO rats have an increased glucose tolerance compared to DR rats following an OGTT test.

When tested for differences in food intake in response to exogenous administration of CCK, we found that both rat strains reduced intake equally in response to 2.0 μg/kg dose of CCK. However, treatment with 4.0 μg/kg dose of CCK produced a significant suppression of chow intake in the DIO rats while DR rats did not suppress intake compared to saline baseline. Previous work by Chandler et. al (17) has shown that outbred DIO rats were more sensitive to a low dose (0.3 μg/kg) of CCK than DR rats; however this was after introduction to a HF diet and only one dose of CCK was tested. In our preliminary tests, using a range of CCK doses (0.1 to 0.5 μg/kg) we found no difference in 30 min chow intake (data not shown). Previous work from our laboratory showed that rats

maintained on a 34% fat diet decreased the satiety action of CCK-8 compared to a 5% fat diet and this effect was due to the amount of fat in the diet and not body weight differences or increased consumption. Torregrossa and Smith reported that rats maintained on 60% fat diet were in fact more sensitive to CCK-8. However, these rats had higher body weights and the increase in sensitivity was related to their hyperphagia. Therefore, it is still not clear what factors may be responsible for differences in CCK sensitivity. In the DIO model, it appears that the amount of fat in the diet may not be responsible for differences in CCK responses since there were no difference between DIO resistant and chow fed control. However, some other physiological factors responsible for the DIO phenotype may also play a role. For example, increased body weight results in increased leptin levels which may be responsible for increased CCK sensitivity, given the interaction between CCK and leptin. Although leptin levels were not measured in this study, the DIO rats had lower fat mass than DR rats, therefore it is unlikely that they had more circulating leptin. This might also explain a slight reduction in sensitivity of DIO rats at the 8 µg/kg dose of CCK. However, the results from Chandler et al. suggest that increased weight gain and leptin levels are not necessary for increased CCK responsitivity. Therefore, more studies are needed to control for body weight and carcass content differences.

The effective doses used in our study, were significantly higher than the ones we typically used in our previous work with regular Sprague-Dawley rats. The observed suppression from baseline for both DIO and DR rats was relatively low compared to previously published results using lower or similar doses (18,

22, 38, 90). One reason for this discrepancy could be explained by the large individual variability within each strain. This was clearly illustrated by the large SEMs resulting in lack of statistical significance between the groups following the 4.0 µg/kg dose of CCK. While there have not been any studies directly correlating body weight to CCK sensitivity, obesity models often exhibit resistance to the satiating effects of CCK (75). However, in the DIO and DR, we did not observe trends between increased obesity and CCK response at any doses we tested.

Finally, in an effort to assess potential differences in vagal transmission in DIO and DR rats, we examined Fos-Li in the dorsal hindbrain, more specifically, the NTS, AP and DMV. Due to its strong correlation with food intake (119), Fos-Li is expected to match the behavioral trends obtained with CCK sensitivity tests While we found that DIO rats had an increased number of fos-positive nuclei in the NTS in response to a 4.0 µg/kg treatment of CCK, we also found that DIO had similar levels of fos in the NTS after a 8.0 µg/kg treatment. Fos-positive nuclei in the NTS is thought to be dose dependent with CCK; however, both 4.0 and 8.0 µg/kg treatments resulted in similar amounts of fos in DIO rats. One possible explanation for this could be an increased CCK-1R number, as suggested by data from Paulino et al. (79). Reduced vagal responses to CCK as observed following the 8 µg/kg CCK dose could result from a down regulation of vagal CCK receptors or the signal transduction cascade coupled to these receptors. Increased plasma CCK concentration could result in desensitization or down regulation of CCK receptors. However, we do not know whether DIO

phenotype is characterized by high CCK levels and no data in the literature suggests a link between body weight and CCK sensitivity. Increase in body fat is normally associated with increased in leptin that enhanced Fos expression following CCK (112). As stated above we did not measure leptin levels in this study, however it is worth mentioning that increased leptin levels would enhance CCK sensitivity as is the case with our effects following a 4.0 µg/kg CCK dose.

In summary, our study shows that DIO rats contain significantly less relative fat pad mass while eating more within a 24 hour period and gaining more body weight than DR when subjected to overnight fasting every other day. At the same time, we have shown that on a chow diet, DIO rats are more sensitive to the satiating effects of CCK at a dose of 4.0 µg/kg compared to DR and that within both strains, there's a large variation in response to CCK, with DIO rats varying less than DR. Further, assessment of Fos-Li in the NTS resulted in DIO rats expressing more Fos-Li than DR in response to a treatment of 4.0 µg/kg dose of CCK.

CHAPTER 4

SUMMARY OF RESULTS, GENERAL DISCUSSION AND PERSPECTIVES

Summary of Results

The overall goal of this thesis was to examine satiation deficits in two rat models of obesity: 1) the Otsuka Long Evans Tokushima Fatty rat, a natural spontaneous mutation that lacks functional CCK-1 receptors, and 2) the dietary induced obesity model (DIO). This was carried out in two specific aims:

Specific Aim 1: *examined the orosensory responses to oils in the presence or absence of gastrointestinal feedback in prediabetic obese OLETF and LETO rats.*

The results presented in Chapter 2 demonstrated that OLETF rats consumed oils of 50 and 75% more than LETO rats while LETO rats consumed more of 12.5 and 25% oils than LETO. Overnight deprivation caused OLETF to drink more of only the 12.5% oil than LETO and 2-hr deprivation resulted in OLETF drinking significantly more 25 and 50% oil than LETO. Despite both OLETF and LETO preferring corn oil to mineral oil while non-deprived, OLETF preferred corn oil to mineral oil while food deprived and LETO preferred each oil equally. Real feeding tests resulted in OLETF consuming significantly more 50, 75, and 100% oils compared to LETO. For all conditions, except non-deprived, within strain comparison revealed OLETF and LETO oil consumption followed an inverted-U pattern with rats consuming more of the lower concentrations. These results showed that OLETF rats have an increased oral sensitivity to oils and will

feed higher oil concentrations while in a fed state. However, deprivation results in a leftward curve shift to consume lower oil concentrations.

Specific Aim 2: *examined feeding and vagal responses to CCK-8 in DIO and DR rats.*

The results depicted in Chapter 3 show that DIO rats are more sensitive to the suppressive effects of CCK-8 in response to 4.0 µg/kg dose of CCK, compared to DR rats. This was accompanied by an increased amount of Fos expression in the dorsal hindbrain. In addition, DR rats consumed more food over a 24h period, gained significantly more weight and had less relative fat pad weights based on their body weight.

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General Discussion and Perspectives

Although the etiology of obesity is complex, there is no dispute that meal size is controlled by orosensory stimulatory and postingestive inhibitory feedback (102). Thus, enhanced responsiveness to orosensory stimulatory properties of a meal, decreased sensitivity to postingestive inhibitory signals or both could result in increased appetite and overeating. Increased palatability of foods that contain fats plays a critical role in increasing the overall calorie intake and can establish obesity. The OLETF rats represent an ideal model to examine mechanisms involved in hyperphagia in the absence of the metabolic derangements associated with hyperphagia. In addition to a host of peripheral satiation deficits such as responses to CCK, gastric and intestinal deficits, OLETF rats have an overall augmented sensitivity for sweet solutions. Previous work from our

laboratory showed that OLETF rats were less sensitive to the intraintestinal infusion of lipids. The mechanisms by which fats are detected are not filly elucidated. Fat and oil ingestion is guided by post-ingestive as well as orosensory factors, so it is conceivable that the rewarding properties of lipid ingestion originate in the gut. Several studies, however, indicate that postingestive factors are not the driving force behind fat and oil preference. Greenberg and Smith (41, 103) compared corn oil and mineral oil ingestion in rats surgically equipped with a stomach fistula that drained the gastric contents as they entered the stomach. Because postingestive factors were virtually eliminated in these sham-fed rats, their oil consumption was driven almost exclusively by orosensory factors. Two groups of rats exposed to either corn oil or mineral oil drank equivalent amounts during the 30-minute test. When the mineral oil animals were subsequently given corn oil, they consumed twice as much as the other group of rats that switched from corn oil to mineral oil. Clearly, both groups of sham-fed rats were able to modify their oil consumption based solely on orosensory information. During a subsequent test in which the two groups of rats had simultaneous access to both oils, they preferred the corn oil. These data show that the orosensory properties are sufficient for discrimination and ingestion of corn oil and mineral oil, independently of postingestive factors.

This study is the first one demonstrating increased sensitivity to oils in OLETF rats. The mechanisms by which this occurs is not clear, however the role of the gustatory system on detection of various oil characteristics remain to be elucidated.

The gastrointestinal feedback including its complex hormonal machinery contributes to meal size. Several reports indicate that reduced sensitivity to anorexic signals could potentially results in overeating and subsequent obesity. Both nutrients and some satiation signals share similar neuronal signaling substrates. This is the case with fats and CCK. For example, suppression of food intake by intraintestinal lipids or CCK-8 is attenuated by CCK -1 receptor antagonists. Therefore, pharmacological intervention using the cholecystokinergic system may be used in curbing appetite. Using the dietary induced obesity rats that resemble the human obesity phenotype, we showed a differential response to CCK. These results open an entire field of studies in this rat model. For example, it is not known whether these changes can be reversible or not when placed on diets with various proportions of other macronutrients. Are DIO rats different in their responses to other satiation signals? What about their sensitivity to oral sensory properties of fats or sweet solutions? What are the physiological and neuronal mechanisms responsible for these differences? What are the signal transductions mechanisms responsible for the prone phenotype? While Levin and other groups have examined differences in the energy metabolism including changes in the hypothalamic peptides responsible for caloric and body weight regulation, there is vey little work done examining deficits in peripheral signaling in this animal model. The work in this thesis barely began to address these questions.

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