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ALTERED STIMULATORY AND INHIBITORY FEEDBACK SIGNALS IN THE CCK-1 RECEPTOR DEFICIENT OLETF RAT

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ABSTRACT

The present studies examine alterations in stimulatory and inhibitory feedback signals in the obese CCK-1 receptor deficient OLETF rat. In the first series of experiments, both gastric emptying of various nutrients and volumes, as well as gastric detection of volumetric distension were assessed to test whether gastric feedback impairments contribute to hyperphagia in the OLETF rat. Our findings showed that OLETF rats exhibit marked differences compared to LETO controls in both feeding and neuronal responses to gastric distension, while no such differences were noted for emptying. Our second set of studies provided evidence of altered orosensory controls of meal size in the OLETF rat. Specifically, OLETF rats showed increased preference and intake of sucrose, even when post-absorptive effects were removed. In addition, OLETF rats showed diminished satiation responses to intestinal sucrose infusions. The next succession of experiments went a step further to show that OLETF rats demonstrate increased conditioned preference for sweet solutions, as well as an increased role of orosensory signaling in the consumption of palatable solutions independent of motivational state. The final segments of this work sought to scrutinize dopamine pathways as a possible substrate responsible for increased orosensory sensitivities in the OLETF. Indeed, OLETF rats exhibited altered functioning acoustic startle responses: a measure known to be mediated by dopaminergic pathways. Furthermore, OLETF rats showed greater reductions in palatable sucrose intake after dopamine receptor antagonism. These data complement work by other laboratories, including our own, by provided evidence of altered central processing of orosensory stimuli in the OLETF rat.

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CHAPTER 1

GENERAL INTRODUCTION

Overweight and obesity in children and adults are continually climbing to epidemic levels in the US, and with its complications may soon become the leading cause of death among adults (Geiss et al. 2006; Ogden et al. 2006). In the face of such a prevalent and growing public health concern, it is essential to investigate possible alterations and impairments on the controls of food intake and body weight regulation that may contribute to the overweight phenotype.

Controls of food intake: The role of CCK and the CCK-1 receptor

Food intake occurs within distinctive sessions, or meals, while the daily patterns of meal consumption constitute meal frequency. The controls of food intake are mediated by multiple signals that determine both meal size and frequency (Smith 1996). Among these factors, negative feedback signals appear to be the most abundant. Signals that arise in the periphery from the gastrointestinal tract in response to nutrient delivery, termed satiation signals (e.g. intestinal nutrient feedback, and peptides such as PYY_{3-36}, and CCK), promote satiation and limit overall ingestion [see (Ritter 2004) for review]. In addition, negative feedback signals which function primarily in longer term regulation of body weight through activation of centrally located receptors, called adiposity signals (e.g. the hormones insulin and leptin), also reduce food intake [see (Woods et al. 2004) for review]. It is clear that the control of food intake is a mechanism governed by a vast

combination of inputs from multiple signaling pathways. One such signal which has been widely studied in the satiation for food is CCK.

Gibbs, Young and Smith first discovered that exogenous administration of CCK caused a dose-dependent decrease in meal size (Gibbs et al. 1973). A vast number of studies have since documented CCK as one of the most biologically potent satiety peptides controlling food intake. CCK reduces food intake primarily through receptor activation on vagal sensory neurons (Smith 1985; South 1988; Schwartz et al. 1993; Moran et al. 1997). Conversely, surgical (Smith et al. 1981; Reidelberger 1992) as well as chemical (Ritter and Ladenheim 1985; South 1988) destruction of vagal sensory fibers abolishes CCK-induced reductions in food intake. Furthermore, CCK receptors are known to be transported via vagal sensory neurons (Moran 1987; Moran 1990). Subsequent studies have identified and cloned the genes encoding these receptors (Kopin et al. 1992; Wank et al. 1992; de-Weerth et al. 1993; Wank et al. 1994). Results of electrophysiological experiments indicate that exogenous CCK can activate vagal sensory fibers innervating the gastrointestinal tract (Ritter and Ladenheim 1985; Blackshaw and Grundy 1990; Schwartz and Moran 1994; Schwartz et al. 1995; Richards et al. 1996). Most importantly, 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 (Smith 1991; Moran et al. 1992; Yox et al. 1992; Brenner and Ritter 1996; Brenner and Ritter 1998). Recent evidence also suggests that in addition to the traditional role of controlling satiation, CCK may act as a primary mediator of specific adiposity signals such as insulin or leptin (Riedy et al. 1995; Emond et al. 1999; Matson

1999), indicating a role for CCK not only in meal termination, but in body weight regulation and mechanisms governing energy balance.

CCK-1 receptor deficient rats are hyperphagic

The Otsuka Long-Evans Tokushima Fatty Rat (OLETF), an outbred strain of the Long Evans Tokushima Otsuka rat (LETO), lacks functional CCK-1 receptor expression (Takiguchi et al. 1997). Consistent with this mutation, little or no CCK-1 receptor mRNA is detected in the OLETF pancreas (Funakoshi et al. 1994), while exogenous CCK fails to induce acinar enzyme secretion previously associated with CCK stimulation (Otsuki et al. 1995; Tachibana et al. 1996). This absence of CCK sensitivity is specific in that OLETF acinar cells do secrete amylase in response to bombesin, carbamylcholine and secretin. In contrast, pancreatic CCK binding in OLETF rats is completely absent (Otsuki et al. 1995). These animals provide an excellent model to study obesity and related metabolic abnormalities, such as Non-Insulin Dependent Diabetes Mellitus (NIDDM) due to their spontaneous manifestation of hyperglycemia and hyperinsulinemia relative to age-matched control, non-mutant LETO rats (Kawano et al. 1992). Also, OLETF rats exhibit accelerated rates of weight gain, beginning at 2-3 post-natal day, which eventually culminate in body weights which are ~40% higher than the control LETO strain (Moran et al. 1998; Schwartz et al. 1999). In terms of behavioral eating patterns, OLETF rats ingest meals larger in size than control LETO rats, a phenomenon most closely associated with a diminished sensitivity to within-meal satiety signals, a trait attributed to the lack of CCK-1 receptors. More specifically, systemic administration of CCK in the OLETF rats has no suppressive effect on food intake (Miyasaka et al. 1994; Moran et al. 1998;

Covasa and Ritter 2001). Furthermore, OLETF rats become resistant to peripheral leptin injections (Niimi et al. 1999). Because of the reported synergistic interaction between CCK and leptin on food intake and body weight (Emond et al. 1999; Matson 1999), defects in CCK signaling could have long term consequences for body weight control. Such a possibility is supported by recent work that indicates OLETF rats exhibit a broad range of satiation deficits in response to intestinal nutrients (Moran et al. 1998; Covasa and Ritter 2001). These animals have also been shown to consume normally preferred foods in excess amounts compared to LETO rats (Tsunoda et al. 1998; Schwartz et al. 1999).

CCK-1 receptors control inhibitory gastric feedback mechanisms

Gastric vagal (Schwartz et al. 1993; Schwartz et al. 1994) as well as pyloric (Moran et al. 1988) CCK receptors mediate CCK's action on suppression of food intake as well as the inhibition of gastric emptying. This negative feedback signal has been shown to be mediated by activation of the CCK-1 type receptor for both solid and liquid nutrients (Green et al. 1988; Liddle et al. 1989; Holzer et al. 1994; Moran et al. 1997). Experiments in non-mutant animals using selective CCK-1 antagonists have shown that blockade of these receptors accelerates gastric emptying (Moran et al. 1997). In addition, Schwartz et al. (Schwartz et al. 1993) observed enhanced and amplified vagal afferent activity due to the presence of a gastric load when CCK was simultaneously administered, implying that CCK and its receptors contribute to mechanoreception of gastric contents. In this regard, the CCK-1 receptor deficient OLETF rat offers an ideal

model to study gastric functions known to be mediated by CCK-1 receptors and at the same time may contribute to the understanding of increased meal-size in this strain.

OLETF rat exhibit altered stimulatory signals that control food intake

Several recent data provide evidence that deficits in peripheral signaling mechanisms may account for the excessive meal-size and food intake observed in OLETF rats (Moran et al. 1998; Schwartz et al. 1999; Covasa and Ritter 2001; Bi and Moran 2002). In contrast, our laboratory's studies with CCK receptor antagonists in OLETF and LETO rats suggest that not all satiation deficits and hyperphagic behaviors in OLETF rats are directly related to the lack of CCK-1 receptors (Covasa and Ritter, 2001). Furthermore, Bi and Moran have recently shown altered central Neuropeptide Y (NPY) signaling within the dorsomedial hypothalamus as another contributor to hyperphagia in these animals in a variety of real feeding paradigms (Bi et al. 2001; Bi and Moran 2002; Bi and Moran 2003; Bi et al. 2003; Bi et al. 2004). In contrast, there are scant data examining altered orosensory functioning in OLETF rats as a possible cause for hyperphagia and subsequent weight gain even though it is well established that orosensory components of the food, independent of post-ingestive effects, can have a significant feed-forward impact on the amount of food consumed. In fact, rats will sham feed sucrose in a concentration dependant manner, when no post-absorptive effects are elicited (Weingarten and Watson 1982; Geary and Smith 1985; Nissenbaum and Sclafani 1987). Examinations of possible neuronal causes of hyperphagia in OLETF rats stimulated by the orosensory properties of highly preferred foods have been largely uninvestigated, beyond one study which observed enhanced gustatory sensitivity to

sucrose relative to LETO controls (Tsunoda et al. 1998). More recent data from our laboratory however, have sought to more systematically assess taste functioning in these animals (De Jonghe et al. 2005; Hajnal et al. 2005).

Dopamine is associated with orosensory components of food reward

The overconsumption of highly preferred, palatable foods, either high in fat or carbohydrate, is a phenomenon evident in the animal literature, as well as in human data (Sclafani 1987; Birch 1999; Rolls 2003). The chronic consumption of such foods in large portions over time has been implicated as a major cause for the weight gain contributing to the increasing trends of obesity (Rolls 2003; Young and Nestle 2003). In non-mutant, lean animals, existing experimental evidence purports central activity of dopamine (DA) as a mediator of food reward mechanisms resultant from orosensory stimulation during ingestion (Hernandez and Hoebel 1988; Phillips et al. 1993). More specifically, transient increases in DA levels within the nucleus accumbens (NAcc) occur in response to ingestion of both liquid and solid foods, as well as in response food deprivation from 1 to 24 hrs (Wilson et al. 1995; Martel and Fantino 1996; Hajnal et al. 2004). A positive correlation also exists between volume or amount of food consumed and the relative increase in NAcc DA magnitude of release (Wilson et al. 1995; Martel and Fantino 1996; Hajnal et al. 2004). Moreover, when orosensory components of a sucrose solution are isolated via sham feeding, intake of palatable sucrose solutions trigger DA release as a function of sucrose concentration (Hajnal et al. 2004). Conversely, when nutrients are infused directly into the stomach (i.e., when orosensory stimulation is removed), via gastric intubation, no detectable rise DA was noted (Wilson et al. 1995; Yang et al.

1997). Most importantly, data linking DA involvement to food reward pathways come from studies using DA receptor antagonists for both D1/D5 (e.g., SCH23390) and D2/D3 (e.g., raclopride) receptor families. These data have clearly shown that in sham feeding rats, intraperitoneal (IP) administration of these DA antagonists reduced sham-fed sucrose intake in a dose-dependent manner, which supports the notion that DA signaling facilitates orosensory effects of sucrose ingestion (Hsiao and Smith 1995; Smith 1995).

CCK-1 receptors modulate dopaminergic signaling

In addition to its role in peripheral satiation signaling, CCK found within specific brain regions can play a modulatory role in the activity of several neurotransmitters, including dopamine (DA). Specifically, CCK stimulates DA release by acting on CCK-1 receptors (Crawley 1991), while activation of the CCK-2 receptor confers an inhibitory effect on DA release (Marshall et al. 1991). In contrast to the ubiquitous presence of CCK-2 receptors throughout the brain, populations of central CCK-1 receptors are only known to exist within limited regions, such as the dorsomedial hypothalamus (Moran et al. 1986; Woodruff et al. 1991), striatum (Graham et al. 1991), with specifically high intensity in the caudo-medial shell of the nucleus accumbens (NAcc) (Heidbreder et al. 1992; Lanca et al. 1998). In the caudal NAcc, DA and CCK are co-released in vivo after administration of drugs that increase DA neuronal firing rate (Crawley 1991; Ladurelle et al. 1994). A similar co-release of DA and CCK in the somatodendritic area of the mesoaccumbens DA system, in the ventral tegmental area (VTA) has been shown to affect DA cell firing rate (Hamilton and Freeman 1995). In OLETF rats, recent data have shown altered central DA signaling in response to psychostimulant manipulations,

suggesting DA signaling abnormalities could contribute to strain differences between these animals (Shilling and Feifel 2002; Feifel et al. 2003). These studies, however, have not assessed this variation in the context of the eating environment.

Overall significance and experimental outline

Increased food intake is necessary for the development of obesity in the OLETF rat. If meal intake is restricted to controls levels (Bi and Moran 2002), or energy expenditure is increased via exercise (Bi et al. 2005), obesity progression in the OLETF is largely curbed or eliminated. Thus, in the absence of hyperphagia, the OLETF does not become obese, unlike other genetically obese rats strains such as the Zucker rat where a lack of hyperphagia does not abolish accumulation of excess body weight (Boulange et al. 1979). For these reasons, the OLETF rat represents an excellent model to study ingestive behaviors within the context of the meal in the development of obesity. Recent work has established multiple levels of deficient satiation responsiveness in the OLETF rat (Moran et al. 1998; Covasa et al. 2001), however meal initiation and cessation are of course governed by both negative and positive feedback mechanisms (Smith 1996). To this end, relatively little attention has been paid to the possible central alterations relating to excessive food intake in the OLETF rat, much less those specific to orosensory properties of palatable foods.

The significance of this proposal lies on three levels. First, it dissects the relative influence of orosensory and post-ingestive properties of palatable food consumption in OLETF rats. Second, it addresses how altered motivational states affect preference for

sweet solutions in CCK-1 receptor deficient rats. Third, it examines participation of the dopaminergic signaling on heightened preference for palatable foods in OLETF rats. . Overall, these studies provide insight into alterations in satiation and orosensory controls of intake in an in vivo model where CCK-1 receptor participation is removed. Assessing the role of other systems which are normally influenced by CCK-1 receptor activation, such as DA signaling pathways, may lead to a better understanding of how food reward deficits affect weight gain and obesity.

The objective of this work was to examine alterations in stimulatory and inhibitory feedback signals in the OLETF rat. These directives were studied under three specific aims. The **first specific aim** focused on the hypothesis that OLETF rats express deficits in CCK-mediated satiation by altered gastric emptying and/or detection of gastric distension. The **second specific aim** assessed whether OLETF rats have increased sensitivity to the orosensory properties of sucrose or lipid that may contribute to hyperphagic eating behavior. The **third specific aim** explored the role of the dopaminergic system as a possible substrate contributing to enhanced palatable food preference and intake in OLETF rats.

Specific Aim 1: *Assess 1) alterations in rates of solid and liquid gastric emptying rates in OLETF and LETO rats; 2) effects of volumetric gastric distension on sucrose sham feeding 3) vagal activation of hindbrain neurons following gastric distension in OLETF and LETO rat.*

Experiments in non-mutant animals using selective CCK-1 antagonists have shown that blockade of these receptors accelerates gastric emptying (Moran et al. 1997). In addition, Schwartz et al. (Schwartz et al. 1993) observed enhanced and amplified vagal

afferent activity due to the presence of a gastric load when CCK was simultaneously administered, implying that CCK and its receptors contribute to mechanoreception of gastric contents. Results of experiments examining the hypothesis that OLETF rats express deficits in CCK-mediated satiation by altered gastric emptying and/or detection of gastric distension are reported in **Chapter 2**.

Specific Aim 2: *Examine whether OLETF rats have increased sensitivity to the orosensory properties of sucrose or lipid that may contribute to hyperphagic eating behavior.*

In contrast to data showing deficits in peripheral signaling mechanisms in the OLETF rat (Moran et al. 1998; Schwartz et al. 1999; Covasa and Ritter 2001; Bi and Moran 2002), scant data examine altered orosensory functioning in OLETF rats as a possible cause for hyperphagia. Data presented in **Chapter 3** explore alterations in sucrose preference, and intake when orosensory components are isolated. Bi and Moran have shown that OLETF rats overconsume chow to a higher degree than LETO rats when previously food deprived for 24 hrs (Bi and Moran 2003). **Chapter 4** shows results examining effect of food restriction on the intake of preferred liquid solutions. **Chapter 4** also contains results of investigations of expression of conditioned flavor preferences for sweet solutions in OLETF and LETO rats under food deprived and non food deprived conditions. Data presented in **Chapter 7** examine possible differential intake of corn oil solutions when post-gastric components of the solution were minimized.

Specific Aim 3: *Explore the role of the dopaminergic system as a possible substrate contributing to enhanced palatable food preference and intake in OLETF rats.*

In addition to its role in peripheral satiation signaling, CCK found within specific brain regions can play a modulatory role in the activity of dopamine (DA). Furthermore, data linking DA involvement to food reward pathways come from studies using DA receptor antagonists for both D1/D5 and D2/D3 receptor families. To indirectly assess altered DAergic functioning in the OLETF rat, **Chapter 5** shows results of examinations in sucrose-receiving OLETF and LETO rats of acoustic startle response and pre-pulse inhibition: behaviors known to be affected by both DA and CCK-1 receptor antagonists. To more directly examine altered DA signaling in the OLETF rat, **Chapter 6** presents results of alterations in sucrose feeding following DA receptor antagonism. **Chapter 7** show results of changes in corn oil intake due to DA receptor blockade.

CHAPTER 2

DECREASED GASTRIC MECHANODETECTION, BUT PRESERVED GASTRIC EMPTYING, IN OLETF RATS

INTRODUCTION

Gastric emptying is one mechanism through which CCK functions to reduce food intake (Moran and McHugh 1982). In non-mutant animals, CCK-1 receptor antagonists have been shown to attenuate inhibition of gastric emptying by both exogenous CCK administration (Green et al. 1988; Moran et al. 1994), as well as gastric nutrient loads (Moran et al. 1997; White et al. 2000). The mechanism for both of these effects appears to be largely mediated through CCK-1 receptor activation of vagal afferents (Schwartz et al. 1993; Schwartz et al. 1994). In addition, Schwartz et al. (Schwartz et al. 1993) observed enhanced and amplified vagal afferent activity due to the presence of a gastric load when CCK was simultaneously administered, implying that CCK and its receptors contribute to mechanoreception of gastric contents. In this regard, the CCK-1 receptor deficient OLETF rat offers an ideal model to study gastric functions known to be mediated by CCK-1 receptors and at the same time may contribute to the understanding of increased meal-size in this strain.

Therefore, in the present study, we investigated whether in addition to defects in intestinal nutrient and CCK satiation signaling deficits, OLETF rats also express impairments in CCK-mediated satiation via defective gastric contributions to meal termination. Accordingly, in the first series of experiments we assessed gastric emptying

rates of solid and liquid foods in OLETF and LETO rats using various feeding conditions and gastric load manipulations.

Diminished detection of gastric volume, due to a lack of gastric vagal afferent CCK-1 receptor activation (Schwartz et al. 1993), may also lead to increased meal size in the OLETF rat. To examine this possibility, the second series of experiments examined the effects of stomach distension on sham feeding and gastric emptying. Finally, gastric distension has been shown to excite specific regions of the dorsal medulla controlling for meal size via vagal activation (Schwartz et al. 1993). Quantification of the immediate-early gene product Fos has been used as an indicator of neuronal activation stemming from vagal afferent transmission of such signaling (Fraser et al. 1995; Willing and Berthoud 1997; Mazda et al. 2004). Thus, the final experiment examined whether or not OLETF and LETO rats differ in neuronal responsiveness to gastric distension by assessing Fos-like immunoreactivity (Fos-LI) in select areas of the hindbrain. To control for possible confounding gastroparetic effects resultant from NIDDM development (Kong et al. 1996) in OLETF rats, experiments were performed in two age groups (12 and 29 wks) representing non-diabetic and pre-diabetic OLETF animals unless otherwise indicated.

METHODS

Subjects

Male OLETF and LETO rats were obtained as a generous gift of the Tokushima Research Institute, Otsuka Pharmaceutical, Tokushima, Japan. Twelve (age1: 423 ± 12.0 g and 315 ± 6.0 g for OLETF and LETO rats, respectively) and twenty-nine (age2: 557 ±

17.0 g and 450 ± 6.4 g for OLETF and LETO rats, respectively) wk old rats were used in these experiments. Separate groups of rats were used for each set of experiments within both age groups tested, unless otherwise indicated. All animals were individually housed in mesh-floored, stainless-steel hanging cages in a temperature-controlled vivarium while maintained on a constant 12:12-h light-dark cycle (lights on at 0600). Rats were handled daily for a minimum of one week prior to the onset of experimental procedures. Tap water and pelleted rat chow (Purina 5001) were available ad libitum throughout the experiments. All protocols used were approved by The Pennsylvania State University Institutional Animal Care and Use Committee.

Procedures

Gastric Fistula Implantation

Rats designated for solid food emptying studies were fasted overnight and anesthetized prior to surgery via intramuscular injection with 1.0 ml/kg of a mixture of ketamine HCl (100.0 mg/ml), xylazine (20.0 mg/ml), and acepromazine maleate (10.0 mg/ml), obtained from Burns Veterinary Supply, Rockville Centre, NY and surgically implanted with chronic gastric fistulae according to Yox and Ritter (Yox and Ritter 1988). The inner flange of a gastric fistula [stainless steel; 13 mm length; 6 mm (ID); 8 mm (OD)] was inserted through the ventral wall of the non-glandular portion of the stomach near the greater curvature and subsequently secured with a purse string suture. A piece of Marlex mesh was centered to adhere flush with the outer flange of the fistula. The non-flanged end of the fistula was then externalized through a left paramedian abdominal incision. A removable stainless steel screw inserted into the fistula blocked access to the stomach lumen between experiments. The peritoneum and abdominal muscles were

simultaneously sealed with absorbable sutures post-implantation. The abdominal skin incision was closed using wound clips, which were removed 7 days post-surgery. Rats were allowed a minimum of one week to recover from fistula implantation surgery before experimentation.

Gastric emptying of a 5 g solid chow meal in OLETF and LETO rats

Due to the known hyperphagic phenotype of the OLETF rat, this experiment controlled for increased meal size by limiting the size of the test meal to a known amount readily consumed by both OLETF and LETO rats within one hour. Overnight (16 hrs) fasted OLETF and LETO rats (n=6-8 per strain, at both age1 and age2) were presented with one, pre-weighed, 5 g pellet of rat chow for consumption. All rats had no food left in the cage before assessment of emptying. Collected spillage was weighed in order to accurately measure how much of the 5 g meal was consumed. Gastric emptying of the chow consumed was measured at 1, 2, or 4 hrs post-presentation. Before the tests, the stainless steel screws occluding the gastric fistulae were removed, and stomach contents were rinsed with warm tap water by continual flushing until the solution withdrawn was clear of ingested chow particles. Both recovered gastric contents and samples of pelleted chow were dried according to procedures previously established in order to isolate dry matter (DM) content (Chelikani et al. 2005). DM emptied (%) was calculated by the following equation: DM emptied (%)= [1 – (dry matter of stomach contents (g)) / (dry matter of food ingested (g))] * 100 (Chelikani et al. 2005). Experiments were conducted a minimum of two occasions, every other day.

Gastric emptying of solid chow in OLETF and LETO rats allowed ad-libitum access to food

The rates of gastric emptying within a meal (i.e., when gastric contents accumulate as a result of ongoing ingestion) have been previously shown to greatly exceed the rate of nutrient emptying following meal termination (Kaplan et al. 1992; van der Velde et al. 1999). Thus, in the hyperphagic OLETF rat, the greater magnitude of gastric fill due to increased meal size may result in increased emptying relative to LETO controls. To investigate this effect, we next allowed animals free access to chow, in contrast to the previous experiment when they were fed with a restricted amount, for either 1, 2 or 4 hrs. After these periods, gastric emptying was measured. This design examined whether increased food consumption by OLETF rats would increase evacuation of gastric contents. Rats used in the previous experiment (n=6-8 per strain, at both age1 and age2) were also used in this study.

Gastric emptying of liquid solutions in OLETF and LETO rats

Since OLETF rats overconsume not only solid but also liquid foods, the following study examined gastric emptying of both isosmotic and hyperosmotic nutritive and non-nutritive liquid loads. To do so, we measured gastric emptying of known liquid loads [isosmotic 0.9% saline (150 mmol/L), isosmotic 5.5% glucose (308 mmol/L), hyperosmotic 2.0% saline (347 mmol/L), and hyperosmotic 12.5% glucose (694 mmol/L)] in OLETF and LETO rats. Each of the four solutions was tested twice in all rats. During testing, 16-hr overnight fasted rats (n= 6-8 per strain, at both age1 and age2) received a 5 ml volume of load containing 0.006% phenol-red instilled into the rat's

stomach via oral gavage. Following a 5 min emptying period, the remaining gastric contents were withdrawn, and the stomach rinsed repeatedly with water until withdraws were void of any visible phenol indicator. Gastric emptying was determined by dye-dilution spectrophotometry from absorption at 550nm as previously described by our laboratory (Hayes et al. 2004). Five minute gastric emptying was determined from the following formula: Liquid emptied (%) = [1 – (phenol red recovered from stomach) / (phenol red in instilled load)] * 100 (Chelikani et al. 2005).

Gastric Emptying of semi-solid chow load in OLETF and LETO rats

For each of the prior solid chow gastric emptying experiments, rats freely ingested their gastric "load" prior to measurement of emptying rates. Based on previous reports by Kaplan et al. (Kaplan et al. 1992) showing that the rate of oral delivery changes the rate of gastric emptying, we elected to test the rate of gastric emptying following direct gastric infusion of a semi-solid chow emulsion, in the absence of oral stimulation. A relatively large load of a semi-solid chow mixture was chosen in order to examine gastric emptying of a load with a high degree of gastric distension, in addition to nutrient content. Specifically, a 15 ml load of semi-solid (25%, wt/v) chow mixture homogenized in distilled water was directly instilled into the stomach through the gastric fistula. The remaining gastric contents were removed at 1 or 2 hrs following instillation of the gastric load and emptying was determined using the DM method as described above.

Sham feeding of sucrose in response to gastric distension in OLETF and LETO rats.

This experiment assessed whether or not OLETF and LETO rats differ in their detection of gastric volume in the absence of post-gastric feedback. To do this, we compared sham feeding of naïve OLETF and LETO rats (n=8 per strain at age1, n=6 per strain at age2) in response to volumetric distension by an intragastric balloon. Following a 16-hr fast, the stainless steel screws occluding the gastric fistulae were removed, and stomach contents were lavaged with warm tap water to ensure minimal gastric volume and distension upon start of sham feeding. Rats were placed into Plexiglas sham feeding boxes and acclimated to the sham feeding procedure by presenting them with 0.3 M (10.26%) sucrose for 90 min over several sessions until stable baseline intake was reached (approximately 3-4 sessions on consecutive days). Subsequently, on the tests, the effects of gastric distension on intake were evaluated. Different degrees of gastric distension were administered using an 8-fr Foley catheter (Bardex, Bard inc., Covington, Ga) with an inflatable tip. Before presentation of sucrose, the inflatable end of the catheter was fed through a drainage tube attached to the gastric fistula and advanced 0.5-0.7cm into the lumen of the stomach. The catheter was held in place by a rubber band attached to the external end which prevented movement from the original insertion position. Five minutes later, rats were presented with burettes filled with 0.3 M sucrose solution. Ten min post-presentation of sucrose, the catheter was inflated (~20 sec inflation time) with either 5 ml or 10 ml warmed 0.9% saline for a period of 20 min. After the catheter was deflated at 30 min (~20 sec deflation time), rats were allowed to sham feed sucrose for an additional 60 min in order to detect any compensatory changes in intake when the effects of distension were removed. Thus, rats had access to 0.3 M sucrose for a total of 90 min. Each distension load was given a minimum of two

experimental days, and always bracketed by a non-distension experimental day when rats did not receive any load during sham feeding. These non-distension days served to assess possible baseline intake shifts due to distension on the previous experimental day. Sham intake was measured to the nearest 0.1 ml every 5 min. In all sham feeding tests gastric drainage was collected in plastic graduated cylinders placed beneath the cages and the volume recorded at experiment termination. In the event that the volume of fluid ingested was greater than the volume of gastric drainage, or if gastric drainage did not occur within 15 s of the start of sham feeding, the data from that subject were discarded on the basis that the gastric fistula was not properly placed or functioning (Yox and Ritter 1988).

Analysis of c-Fos expression in the hindbrain of OLETF and LETO rats following gastric distension.

A separate group of OLETF and LETO rats were used for analysis of Fos expression in response to gastric distension. Overnight (16 hr) food-deprived OLETF and LETO rats were removed from their home cages and placed in Plexiglass sham-feeding boxes as described in the preceding experiment. Twenty min following attachment of the drainage tube, an 8-Fr Foley catheter was inflated with warm tap water as in the previous experiment. Eight rats (n=4 per strain) had their stomachs distended with 8 ml of warmed water for a period of 90 min, while 6 rats (n=3 per strain) underwent all procedures as above except that no inflation of the catheter occurred (sham distension). These methods have been previously described by van de Wall and colleagues with slight modifications (van de Wall et al. 2005).

Ninety minutes following the onset of gastric distension, all animals were deeply anesthetized and intracardially perfused using a 0.1 M phosphate buffer solution, followed by 4% paraformaldehyde in 0.1 M phosphate buffer, (pH 7.4). Whole brains were then removed, subsequently stored for 4 hrs in 4% paraformaldehyde, and finally transferred to 20% sucrose solution for overnight storage. Thirty-µm cryostat cut sections were processed for Fos-like immunohistochemistry (Fos-LI) as previously described (Covasa and Ritter 2005). Stained brain sections were inspected microscopically and counts of all Fos-LI nuclei were made. The counts were done manually by an individual blinded to the treatments. Fos-LI nuclei were counted bilaterally, in the dorsal vagal complex (DVC) that comprised the nucleus of the solitary tract (NTS), area postrema (AP), area subpostrema (AsP), and dorsal motor nucleus of the vagus (DMV), at six levels of the dorsal hindbrain (−14.30 mm, -14.08 mm,−13.80 mm, -13.68 mm, -13.30 mm and −13.24 mm from bregma) corresponding to plate levels 76-71 according to the stereotaxic atlas of Paxinos and Watson (Paxinos 1998). At minimum, three sections per each brain level were analyzed for each rat. The presented data are the average number of Fos-LI cells within or across plate level for each rat and treatment condition.

Determination of stomach weights

OLETF and LETO rats used in experiments at age2 (n=16 per strain) were sacrificed and stomachs harvested after study completion. Briefly, the stomach was exposed via a midline celiotomy, ligated at the pylorus and cardia, resected, and weighed. The resected stomachs were then incised and scraped clean of any food particles. The empty stomachs were blotted to remove excess liquid and weighed (Hayes et al. 2004).

Oral Glucose Tolerance Test (OGTT) and Insulin Tolerance Test (ITT)

An oral glucose and insulin tolerance test were performed in a subset of rats (n=7 per strain) within each age group after experimentation. For the OGTT, after a 16hr fast, an oral glucose load (2 g/kg) was delivered to each rat orally via latex gavage. For ITT, human regular insulin (0.75 U/kg body weight: Humalin R; Eli Lilly Japan K.K., Kobe, Japan) was administered intraperitoneally (IP) to all rats. For both tests, blood glucose was measured before gavage or pre-injection and at 30, 60, 90, and 120 min post-glucose loading or insulin injection by a standard glucometer (LifeScan, One-Touch Basic). Animals were classified as diabetic if the peak level of plasma glucose was ≥300 mg/dL and a peak glucose level at 120 min > 200 mg/dL (Kawano et al. 1992).

Statistical Analysis

For solid gastric emptying experiments, gastric emptying was analyzed using two way repeated measures analyses of variance (rmANOVA) with strain and time as main factors. Food intake was analyzed using one or two way rmANOVAs where applicable. Liquid gastric emptying was examined using two way rmANOVAs with strain and gastric load as main factors. Gastric emptying of solid and semi-solid chow is presented as percentage dry matter (DM) emptied from the stomach.

For sham feeding/gastric distension experiments, separate two way rmANOVAs were used to calculate effects of distension treatments on individual 5-min intake bins in both OLETF and LETO rats using distension volume and time as main effects. Quantification of Fos-LI nuclei was analyzed by two-way ANOVA with distension

treatment (gastric distension vs. sham) and strain as main factors. Blood glucose in OLETF and LETO rats following an OGTT or ITT test were compared using planned t-tests. For all experiments, ANOVA results were subsequently analyzed by Tukey's honestly significant difference (HSD) post-hoc tests when appropriate. All data were expressed as means + SEM. Differences were considered statistically significant if $P<0.05$. Statistical analyses were computed with PC-SAS (version 8.02, SAS Institute, Carey, NC).

RESULTS

Gastric emptying of a 5 g solid chow meal in OLETF and LETO rats

Both OLETF and LETO within the two age groups consumed the entire 5 g chow meal presented, barring spillage. No strain differences in intake were noted at age1 (4.4 ± 0.1 g and 4.3 ± 0.1 g, for OLETF and LETO rats, respectively; $F(1,15)=0.7$, P=ns) or age2 (4.7 ± 0.1 g and 4.7 ± 0.1 g, for OLETF and LETO rats, respectively; $F(1,15)=0.1$, P=ns). ANOVA results at age1 showed no significant strain x time interaction [$F(2,30)=1.2$, P=ns]. Gastric emptying increased across time [$F(2,30)=25.0$; $P<0.001$], however this was not significantly different between OLETF and LETO rats [$F(1,15)=0.8$, $P=0.877$] at either 1 hr (40.2 ± 5.8 % and 41.1 ± 4.1 %; respectively), 2 hrs (55.2 ± 3.0 % and 60.2 ± 2.2 %; respectively), or 4 hrs (66.5 ± 2.1 % and 68.3 ± 2.5 %; respectively), post-presentation of a 5 g chow meal. Similarly at age2, no significant interaction effect for strain x time was observed [$F(2,30)=0.5$, P= ns], while a significant main effect for time was noted [$F(2,30)=33.5$,$P<0.001$]. At age2, gastric emptying was

again not significantly different between OLETF and LETO rats [F(1,15)=0.6, P=ns] at either 1hr (41.6 ± 3.2 % and 41.8 ± 4.5 %; respectively), 2 hrs (53.4 ± 2.3 % and 50.5 ± 3.0 %; respectively), or 4 hrs (68.7 ± 1.9 % and 68.1 ± 1.8 %; respectively), post-presentation of a 5 g chow meal.

Gastric emptying of solid chow in OLETF and LETO rats allowed ad-libitum access to food.

As shown in Figure 2.1A, when given *ad libitum* access to chow for 1 hr after an overnight fast at age1, OLETF rats consumed more food than LETO rats (F(1,15)=19.0, P<0.001). Gastric emptying ANOVA analyses revealed no significant strain x time interaction [F(2,30)=0.4, P=ns], while a main effect for time [F(2,30)=42.3,P<0.001], but not strain [F(1,15)=0.3, P=ns], was shown.

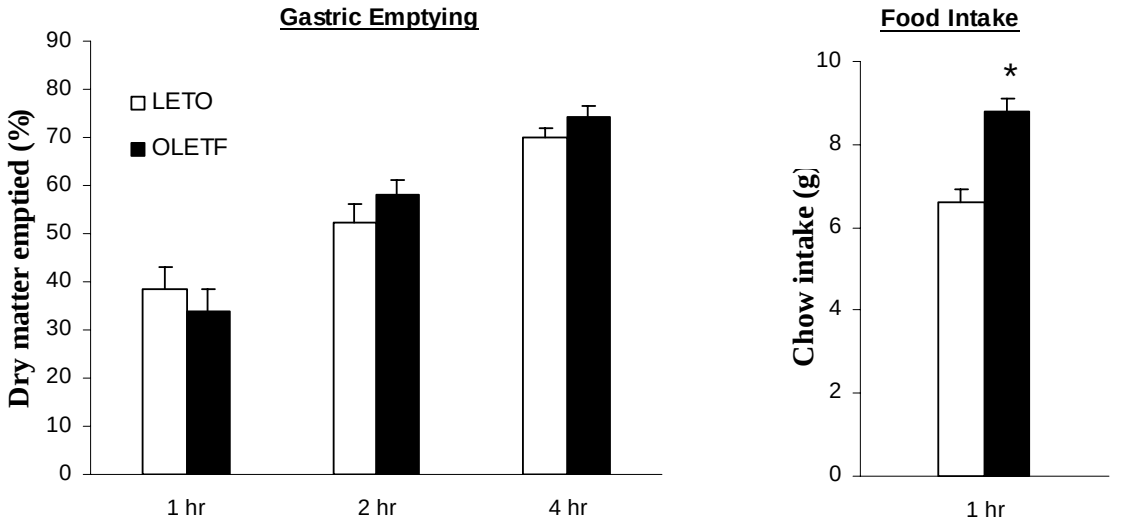
Figure 2.1B shows results from rats at age2 following *ad libitum* access to chow for 1 hr after an overnight fast. As expected, OLETF rats consumed more food than LETO rats (F(1,15)=16.3, P<0.01). Gastric emptying of chow showed a main effect of time [F(2,30)=51.7; P<0.001] but not strain [F(1,15)=0.2, P=ns]. No interaction between strain x time on gastric emptying after 1hr *ad libitum* feeding was observed at age2 [F(2,30)=0.4, P=ns].

When rats were given a larger window of *ad libitum* access to chow at age1, OLETF rats consumed significantly more chow than LETO rats [F(1,15)=13.8, P<0.001] across time [F(2,30)=33.9, P<0.001], however no strain x time interaction was noted [F(2,30)=0.6, P=ns]. Post-hoc results show (Figure 2.2A) increased chow intake in OLETF rats at 1hr (P<0.001), 2 hrs (P<0.05) and 4 hrs (P<0.01) access periods. At age1,

no main effect for strain [F(1,15=0.2, P=ns] on gastric emptying was observed (Figure 2.2A) although a significant time effect was shown [F(2,30)=20.1, P<0.001]. No significant strain x time interaction [F(2,30)=0.2, P=ns] was evident for *ad libitum* chow gastric emptying at age1.

At age2, ANOVA results again showed significant main effects for both strain [F(1,15)=26.9, P<0.001] and time [F(2,30)= 31.8, P<0.001] on chow intake following *ad libitum* access periods, although no strain x time interaction [F(2,30)=0.2, P=ns]. Post-hoc results showed that OLETF rats consumed more chow than control LETO rats at 1 hr, 2 hrs, and 4 hrs (all P's<0.01) access periods (Figure 2B). Figure 2B also illustrates that no significant main effect of strain [F1,15)=0.2, P=ns] was observed for gastric emptying at age2, however a main effect for time on gastric empting was shown F(2,30)=18.3, P<0.001].

A



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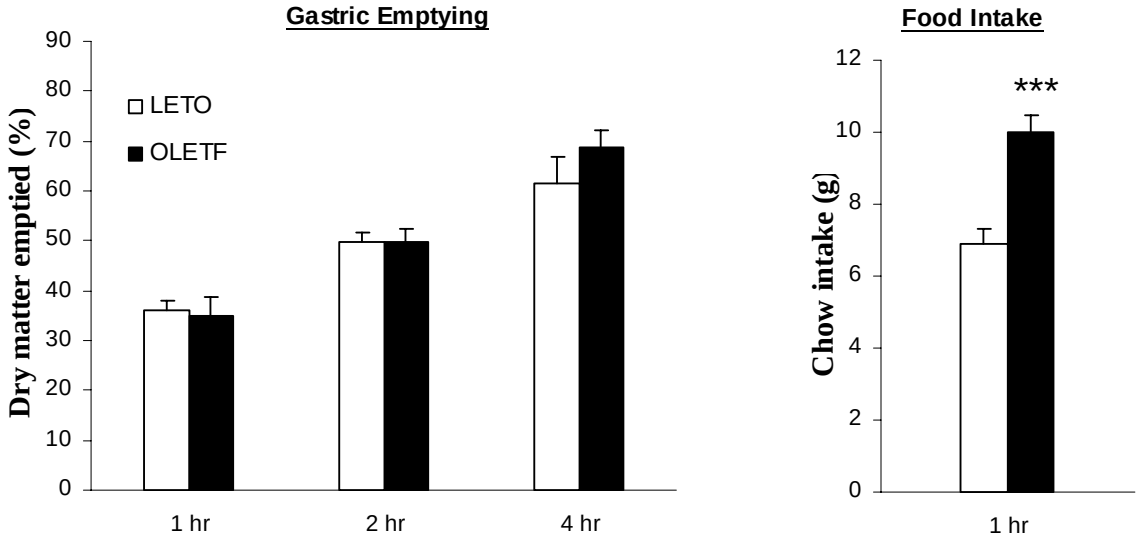
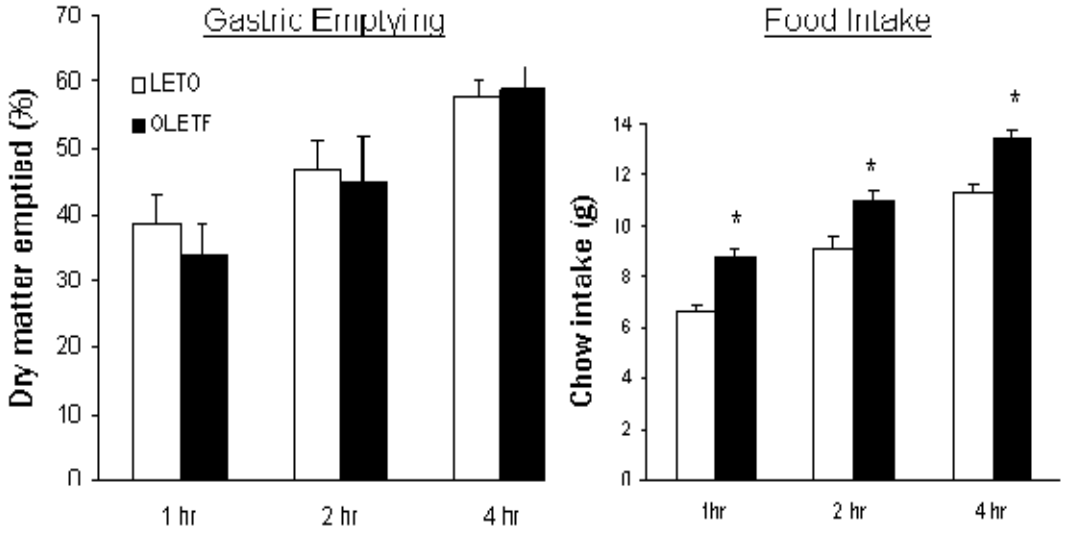


Figure 2.1 Gastric emptying of solid chow in OLETF and LETO rats allowed 1 hr *ad libitum* access to food. Sixteen hour food deprived OLETF and LETO rats were given 1 hr to consume solid rat chow *ad libitum*. Gastric emptying was not different between strain at 1, 2, or 4 hrs after presentation of food at age1 (**A, left panel**) or age2 (**B, left panel**), however, OLETF rats consumed significantly more chow than LETO rats at both age1 (**A, right panel**) and age2 (**C, right panel**). (*P<0.01, *** P<0.001, between strains).

A



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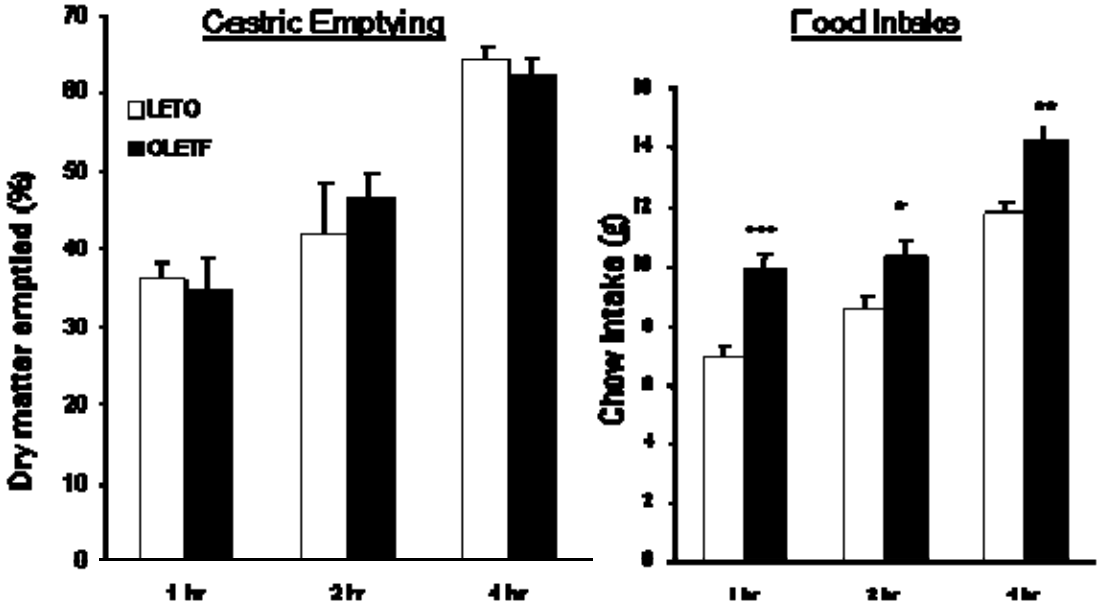


Figure 2.2: Gastric emptying in OLETF and LETO rats allowed variable *ad-libitum* solid chow access. Sixteen hour food deprived OLETF and LETO rats were allowed ad libitum access to rat chow for either 1, 2 or 4 hrs. Gastric emptying was not different between strain immediately following the termination of the chow access period , regardless of duration at both age1 (**A**, left panel) and age2 (**B**, left panel), however, OLETF rats consumed significantly more chow than LETO rats during all periods of ad libitum access at age1 **A**, right panel) and age2 (**B**, right panel) . (*P<0.05, ** P<0.01, *** P<0.001, between strains).

Gastric emptying of liquid loads in OLETF and LETO rats.

No main effects of strain [age1: F(1,15)=0.1, P=ns; age2: F(1,15)=0.2, P=0.945] or gastric load [age1: F(1,15)=0.4, P=ns; age2: F(1,15)=0.4, P=ns] were noted in 5 ml emptying of liquid loads among either age group tested. Specifically, gastric emptying of a 5 ml load of isosmotic saline (age1: 76.2 ± 2.0 % and 72.2 ± 3.7 %; for OLETF and LETO rats, respectively: age2: 59.1 ± 2.0 % and 62.5 ± 2.8 %; for OLETF and LETO rats, respectively), hyperosmotic saline (age1: 59.3 ± 3.9 % and 62.2 ± 4.6 %; for OLETF and LETO rats, respectively: age2: 49.2 ± 2.0 % and 50.5 ± 4.4 %; for OLETF and LETO rats, respectively), isosmotic glucose (age1: 59.5 ± 3.6 % and 56.3 ± 2.8 %;for OLETF and LETO rats, respectively: age2: 50.7 ± 3.3 % and 52.7 ± 4.1 %; for OLETF and LETO rats, respectively), or hyperosmotic glucose (age1: 48.2 ± 5.3 % and 44.5 ± 3.3 %; for OLETF and LETO rats, respectively: age2: 44.2 ± 2.7 % and 42.4 ± 4.6 %; for OLETF and LETO rats, respectively) were no different between strains.

Gastric emptying of semi-solid chow in OLETF and LETO rats.

No significant main effect of strain [F(1,10)=1.1, P=ns] in gastric emptying of a 15 ml load of 25% chow mixture load was noted at age 2 between OLETF and LETO rats at either 1 hr (35.7 ± 4.2 % and 32.6 ± 2.4 %; for OLETF and LETO rats, respectively) or 2 hrs (52.9 ± 3.4 % and 46.1 ± 3.1 %, for OLETF and LETO rats, respectively).

Sham feeding of sucrose in response to gastric distension in OLETF and LETO rats.

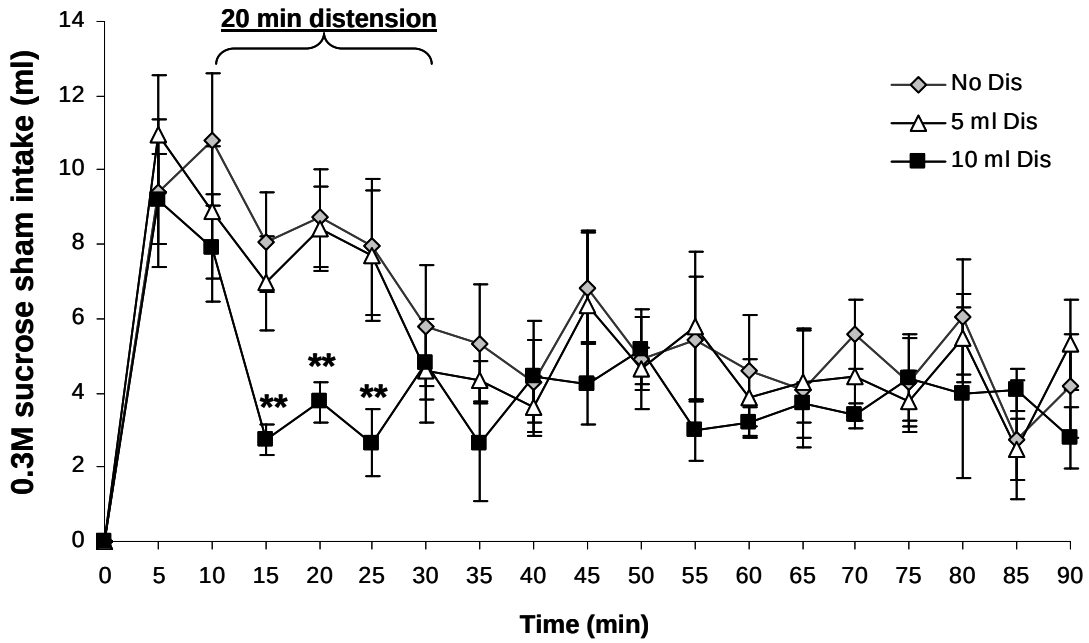
Figures 2.3 and 2.4 depict 20 min gastric distension effects on sham intake of 0.3 M sucrose within five-min bins over a 90 min sham feeding session in OLETF or LETO rats at age1 (Fig. 2.3) or age2 (Fig. 2.4). Figure 2.3A shows results of gastric distension in OLETF rats at age1. Two way ANOVA results showed significant main effects for gastric distension [$F(2,21)=12.3$, $P<0.001$] and time [$F(17,357)= 8.8$, $P<0.01$], as well as a significant distension x time interaction [$F(34,357)=3.54$, $P<0.01$]. Post-hoc analyses of these results show the effects on sham intake to be confined to time periods where distension occurred. Specifically, no response to 5 ml distension was noted in OLETF rats at age1, while significant suppressions in sham intake relative to baseline were noted during 10 ml distension conditions at 15 min , 20 min, and 25 min time points ($P<0.01$ for all three time points).

Figure 2.3B shows age1 results in LETO rats receiving 20 min gastric distension. Two way ANOVAs in LETO rats at age1 reveal significant main effects for both gastric distension [$F(2,21)=24.1$, $P<0.001$] and time [$F(17,357)= 14.3$, $P<0.001$], and also a significant distension x time interaction [$F(34,357)=7.7$, $P<0.001$]. Post-hoc analyses of these results depicted in Figure 2.3B show significant reductions in sham intake only within the 20 min distension period. However unlike in OLETF rats, LETO rats showed significant suppression in intake in response to both 5 ml and 10 ml distension volumes. In particular, intake reductions were noted at 15 min ($P<0.05$ and $P<0.001$, for 5 ml and 10 ml distension, respectively), 20 min ($P<0.01$ and $P<0.001$, for 5 ml and 10 ml distension, respectively), and 25 min ($P<0.05$ and $P<0.01$, for 5 ml and 10 ml distension, respectively), time points.

Gastric distension effects on sham intake in OLETF rats at age2 are shown in Figure 2.4A. Similar to results in age1, OLETF rats at age2 showed significant main effects of distension [$F(2,15)=15.7$, $P<0.001$] and time [$F(17,255)= 14.3$, $P<0.001$], and in addition, a significant distension x time interaction [$F(34,255)=2.6$, $P<0.01$]. Intake at the 15 min time point was significantly reduced in both 5 ml ($P<0.05$) and 10 ml ($P<0.01$) distension conditions, while intake at 20 min was significantly reduced ($P<0.01$) in the 10 ml distension condition only.

Figure 2.4B illustrates effects of gastric distension on sham intake in LETO rats at age2. LETO rats at age2 showed significant main effects of distension [$F(2,15)=26.8$, $P<0.001$] and time [$F(17,255)= 45.2$, $P<0.001$], and a significant distension x time interaction [$F(34,255)=11.9$, $P<0.001$]. Unlike in OLETF rats, post-hoc analyses in LETO rats showed intake reductions during distension at three time points during distention:15 min ($P<0.05$ and $P<0.001$, for 5 ml and 10 ml distension, respectively), 20 min ($P<0.01$ and $P<0.01$, for 5 ml and 10 ml distension, respectively), and 25 min ($P<0.05$ and $P<0.01$, for 5 ml and 10 ml distension, respectively).

A OLETF (age1)



B LETO (age1)

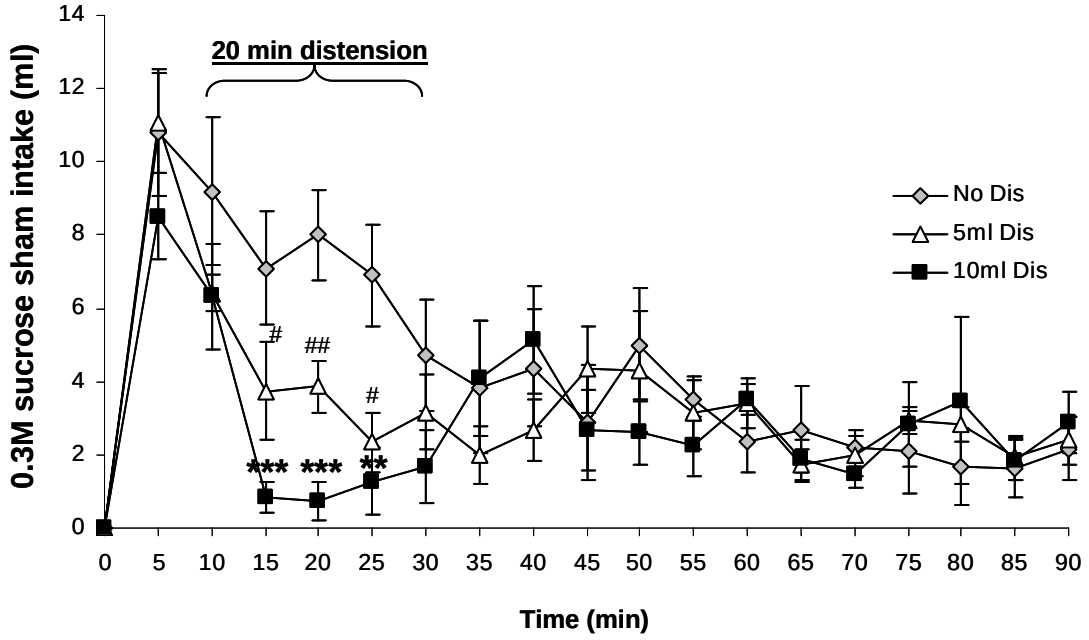
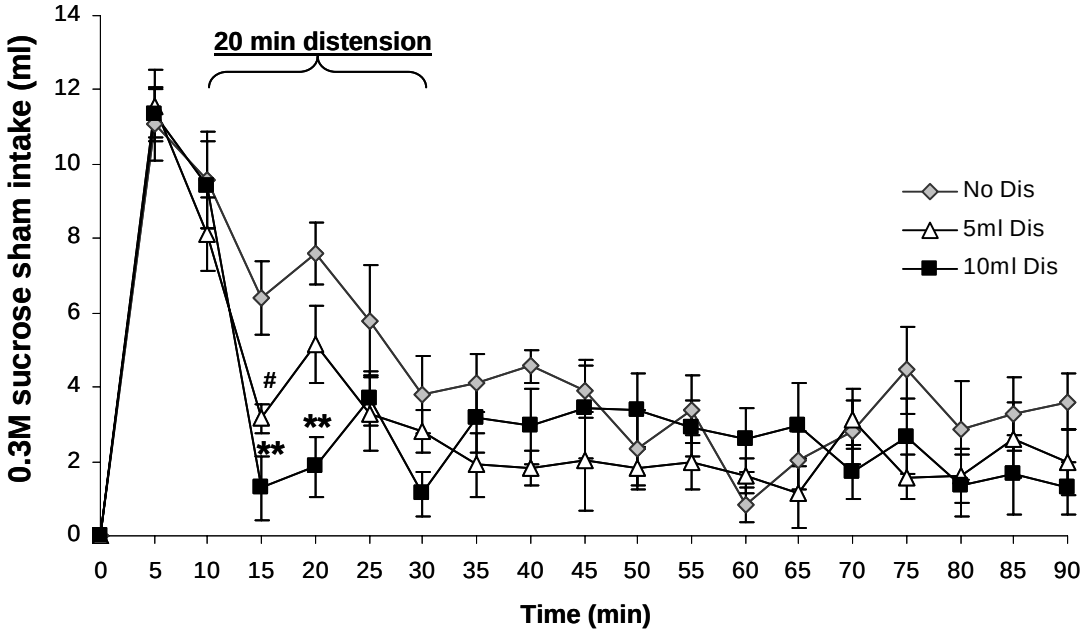


Figure 2.3: Twenty min gastric distension effects on sham intake of 0.3 M sucrose within five-min bins over a 90 min sham feeding session in OLETF or LETO rats at age1. OLETF rats showed significantly attenuated sham intake at age1 during 10 ml distension relative to baseline, no distension conditions at 15, 20, and 25 min time points. No effect on sham intake due to 5 ml distension was observed in OLETF rats at age1 (**A**). In contrast, sham intake in LETO rats was significantly smaller after both 5 and 10 ml distension relative to baseline intake at 15, 20, and 25 min time points at age1 (**B**).. $^{\#}P<0.05$, $^{\#\#}P<0.01$, between no distension and 5 ml distension intake; **P<0.01, ***P<0.01, between no distention and 10 ml distension intake

A OLETF (age2)



B LETO (age2)

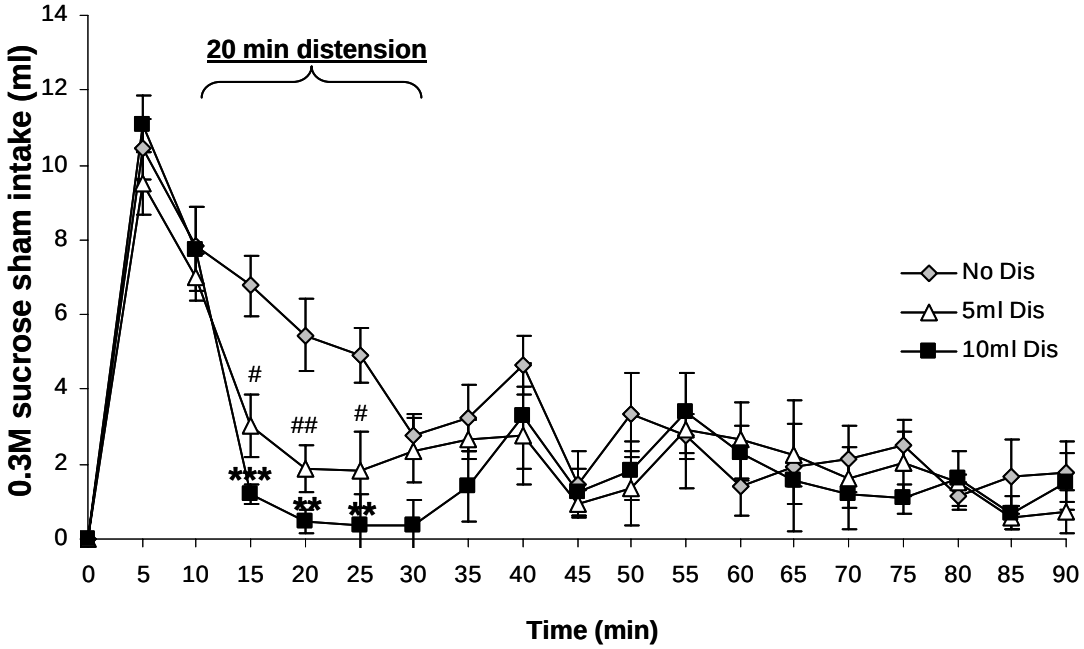


Figure 2.4: Twenty min gastric distension effects on sham intake of 0.3 M sucrose within five-min bins over a 90 min sham feeding session in OLETF or LETO rats at age2. At age2, sham intake in OLETF rats was significantly decreased during 5 ml distension when compared to baseline intake only at 15 min, while intake during 10 ml distension was decreased at 15, 20, and 25 min compared to non distension intake (**A**). In LETO rats at age2, sham intake was significantly lower than under baseline conditions at 15, 20, and 15 min during both 5 and 10 ml distension conditions (**B**). $^{\#}P<0.05$, $^{\#\#}P<0.01$, between no distension and 5 ml distension intake; **P<0.01, ***P<0.01, between no distention and 10 ml distension intake

Analysis of c-Fos protein expression in the hindbrain of OLETF and LETO rats due to gastric distension.

Analyses of c-Fos expression in the hindbrain of OLETF and LETO rats revealed significant differences within the NTS region of the dorsal vagal complex. Specifically, a strain main effect was noted for both sham [$F(1,4)=22.1$; $P<0.01$] and 8 ml distension [$F(1,6)=6.94$; $P<0.05$] conditions, indicating decreased average of NTS Fos-LI in OLETF rats relative to LETO rats (Table 2.1). Post-hoc analyses within NTS plate level showed significant differences between OLETF and LETO rats. Specifically, OLETF rats distended with an 8 ml gastric balloon showed a significant decrease in NTS Fos expression at −13.24 mm ($P<0.05$) and -13.68 mm ($P<0.01$) levels compared to LETO controls. In non-distended sham conditions, OLETF rats showed a decreased Fos expression at −13.30 mm ($P<0.05$) and -13.68 mm ($P<0.05$) levels relative to LETO rats.

There were no significant differences in Fos expression between OLETF and LETO rats within either treatment group at the −14.30 mm (P=ns), -14.08 mm (P=ns), or −13.80 mm (P=ns) plate levels. Additionally, we did not observe any significant differences in Fos expression in any other area of the dorsal vagal complex including the dorsal motor nucleus, the area postrema, or area subpostrema, between OLETF and LETO rats at any plate level examined (all P's>ns).

Plate level	*NTS FOS-LI nuclei*			
	OLETF Distension	**LETO Distension**	**OLETF Sham**	**LETO Sham**
-14.30 mm	42.7 ± 4.5	44.1 ± 2.7	4.6 ± 0.7	6.5 ± 2.1
-14.08 mm	108.2 ± 14.3	101.9 ± 5.9	3.0 ± 0.9	5.4 ± 1.4
-13.80 mm	119.5 ± 8.5	132.4 ± 16.5	3.6 ± 1.8	6.8 ± 2.3
-13.68 mm	121.2 ± 22.9	216.2 ± 17.5**	6.9 ± 1.7	12.8 ± 0.7*
-13.30 mm	156.0 ± 28.4	162.3 ± 14.2	5.6 ± 0.9	14.3 ± 3.7*
-13.24 mm	57.7 ± 8.2	102.5 ± 15.7*	3.9 ± 1.6	5.6 ± 0.9
average	129.7 ± 15.9	164.4 ± 8.7*	4.8 ± 1.6	10.9 ± 0.4**

Table 2.1: Counts of Fos-LI nuclei within the NTS of 8 ml gastric distended or sham distended OLETF and LETO rats. Quantification of Fos-LI in the hindbrain of OLETF and LETO rats. OLETF rats showed decreased average NTS Fos-LI nuclei for both sham and 8 ml distension conditions relative to LETO rats. Post-hoc analyses within NTS plate level showed that OLETF rats distended with an 8 ml gastric balloon showed decreased NTS Fos expression at −13.24 mm and -13.68 mm levels compared to LETO controls, while in non-distended sham animals, OLETF rats showed decreased Fos expression at −13.30 mm and -13.68 mm levels. *P<0.05, **P<0.01 between OLETF and LETO rats within distension or sham distension conditions.

Oral Glucose (OGTT) & Insulin (ITT) Tolerance Tests

As shown in Table 2.2, at both age1 and age2, OLETF rats showed increased blood glucose levels relative to LETO rats after glucose challenge. At age1, significant increases were noted in OLETF rats at 30 min and 60 min ($P<0.001$ for both time points) compared to LETO rats, with highest blood glucose peak at 30 min (173 ± 5.1 vs. 110 ± 5.5 mg/dl in OLETF and LETO rats, respectively). At age2, all time points measured post-glucose challenge were significantly higher in OLETF rats ($P<0.01$ for all time points), with highest blood glucose peaks occurring at 60 min (253 ± 30.0 vs. 111 ± 5.9 mg/dl in OLETF and LETO rats, respectively).

After acute insulin injection at age1, OLETF rats show an attenuated decrease in blood glucose 120 minutes post-insulin injection compared to LETO animals (70 ± 3.5 vs. 51 ± 1.5 mg/dl in OLETF and LETO rats, respectively; $P<0.05$) (Table 2.2). At age2, OLETF rats showed significantly higher blood glucose at 90 min post-insulin injection when compared to LETO rats (53 ± 4.1 vs. 35 ± 2.1 mg/dl in OLETF and LETO rats, respectively; $P<0.01$), as well as a non-significant trend of an overall attenuation of decreased blood glucose due to insulin relative to LETO rats.

Test	*Blood glucose levels (mg/dl)*			
	OLETF age1	**LETO age1**	**OLETF age2**	**LETO age2**
OGTT				
Baseline	86.7 ± 2.2	88.2 ± 2.9	81.5 ± 5.5	82.2 ± 1.5
30 min	173.0 ± 5.1	110.0 ± 5.5***	194.3 ± 20.6	110.2 ± 5.8***
60 min	161.3 ± 4.8	105.6 ± 2.4***	253.2 ± 30.0	111.9 ± 5.9***
90 min	127.3 ± 15.5	99.0 ± 2.1	207.3 ± 20.3	101.8 ± 4.8***
120 min	92. 8 ± 2.3	88.8 ± 2.0	140.0 ± 11.5	91.8 ± 3.8***
ITT				
Baseline	84.4 ± 3.2	79.7 ± 2.4	83.3 ± 2.3	77.3 ± 1.3
30 min	72.3 ± 3.0	72.3 ± 2.0	69.4 ± 4.4	59.5 ± 6.4
60 min	63.5 ± 3.5	63.4 ± 6.0	55.9 ± 5.0	48.8 ± 4.4
90 min	67.6 ± 4.3	57.7 ± 1.7	53.4 ± 4.9	35.0 ± 1.8**
120 min	70.0 ± 5.2	51.0 ± 1.5*	46.3 ± 4.5	39.7 ± 3.7

Table 2.2: Blood glucose levels (mg/dl) following OGTT or ITT in OLETF and LETO rats at age1 and age2. OGTT and ITTs were performed within each age group after experimentation. For OGTT, a glucose load (2g/kg) was orally delivered after a 16 hr fast. For ITT, all rats received intraperitoneal (IP) injection of insulin (0.75 U/kg). For both tests, blood glucose was measured before glucose loading or pre-injection and at 30, 60, 90, and 120 min post-loading or injection. *P<0.05, **P<0.01, ***P<0.001, strain differences in blood glucose levels within each age group tested.

Determination of stomach weight

Following OGTT and ITT administration at age2, animals were sacrificed and stomachs resected. OLETF rat stomachs were significantly heavier than LETO stomachs [$F(1, 30)= 35.4$; $P<0.01$: 2.6 ± 0.1 g vs. 2.0 ± 0.1 g for OLETF and LETO rats, respectively). However when corrected for body weight (BW: 665.1 ± 16.0 g vs. 547 ± 11.5 g for OLETF and LETO rats), the relative stomach size of OLETF and LETO rats

did not differ (stomach weight: 0.39 ± 0.02 vs. 0.37 ± 0.02 g/100 g BW for OLETF and LETO rats, respectively; P=ns).

DISCUSSION

The present findings indicate that OLETF rats do not show deficits in gastric emptying of either solid or liquid loads relative to LETO rats, regardless of age and progression of blood glucose impairments. Specifically, when OLETF rats consumed either a chow meal of equal or greater size within the same allotted meal period, gastric emptying was equal to that of LETO controls. Similarly, applying gastric distension via intragastric instillation of a relatively high volume load did not produce differential gastric emptying between strains. OLETF and LETO rats were also shown to have equal gastric emptying of both isosmotic and hyperosmotic nutritive and non-nutritive gastric loads across both age groups tested. In contrast, when the volumetric effects of gastric mechanodetection were isolated by inflation of an intragastric balloon, we observed reduced feeding responses within OLETF rats relative to LETO in both young and older animals. Furthermore, after gastric distension alone, OLETF rats show marked decreases in Fos expression compared to LETO rats in select regions of the hindbrain known to facilitate the vagal response to changes in gastric volume.

Our gastric emptying data appear to contrast with the expected outcome of accelerated gastric emptying in a CCK-1 receptor deficient animal. An increased rate of emptying would be predicted according to the known role of exogenous CCK to inhibit gastric emptying of solid and liquid nutrients (Dockray 1982; Moran and McHugh 1982; Schwartz et al. 1991; Hayes et al. 2004) via CCK-1 receptors, as well as from studies

observing heightened gastric emptying of liquid nutrients by acute CCK-1 receptor blockade in normal rats (Raybould and Holzer 1992; Moran et al. 1997).

Data by other laboratories have shown decreased duodenal lipid-induced gastric acid secretion (Shoji et al. 1996), and increased susceptibility to gastric mucosal lesions in the OLETF rat (Okumura et al. 1996). More relevant to the current work, however, is that OLETF rats displayed delayed gastric emptying of a methylcellulose load in comparison to LETO rats (Shoji et al. 1997). In that report, the authors showed no differential strain reduction in gastric emptying due to acute administration of corticotropin-releasing factor and the muscarinic receptor antagonist atropine. A closer inspection of the magnitude of these effects, however, shows a much larger, and almost complete, abolishment of gastric emptying in the OLETF rat from atropine (Shoji et al. 1997). It is not clear from the analysis reported whether this increased degree of suppression was significantly higher in OLETF rats. Such a scenario would be suggestive of decreased parasympathetic control of gastric emptying in the OLETF, and may explain these observations.

Ohta et al. extended these findings by showing that OLETF rats exhibit delayed gastric emptying in response to a caloric liquid gastric load (Ohta et al. 2000). It was also theorized that sympathetic nervous function may be enhanced in the OLETF compared to LETO animals as indicated by decreased responsiveness to reserpine-induced gastric emptying acceleration (Ohta et al. 2000). Nonetheless, no studies to date have directly examined the possibility of altered gastric parasympathetic or sympathetic innervation in the OLETF rat.

In the present study, gastric emptying of both caloric and non-caloric, as well as hyperosmotic and isosmotic, gastric loads were no different between OLETF and LETO rats, indicating that fluid emptying in the OLETF rat is also intact. It is worth noting that gastric emptying of a non-nutrient liquid load in CCK-2 receptor knock-out mice is enhanced (Miyasaka et al. 2004), although the precise mechanism behind this phenomenon is not known. Furthermore, gastric CCK-2 receptor mRNA in OLETF rat has been shown to be up-regulated relative to LETO rats (Miyasaka et al. 1998). Therefore, the possibility that putative deficits in gastric emptying in the OLETF rat due to the lack of CCK-1 receptor are compensated for by enhanced CCK-2 receptor activation cannot be discounted.

When a large liquid chow mixture was directly instilled into the stomach, we did not detect any differences in gastric emptying amongst OLETF and LETO rats. It has previously been reported that OLETF rats tend to have higher wet weights of stomachs at 12-14 weeks of age (Ohta et al. 2000), which parallels their increased body weight. We have extended these findings by observing slightly increased dry stomach weights in OLETF rats at ~35 weeks of age (age of sacrifice after age2), however when correcting for body weights of the animals at this age, this difference becomes negligible. Nonetheless, despite this apparent increased raw gastric size, our relatively large 15 ml load was not able to produce any distinguishable difference in gastric emptying between OLETF and LETO rats, suggesting that OLETF rats are able to maintain normal gastric emptying rates of a caloric load even when given a volume of distension likely close to the maximum gastric capacity under ad libitum feeding.

Additionally, Schwartz et al. (Schwartz et al. 1999) have shown that liquid nutritive gastric preloads are largely equal in their ability to suppress subsequent food intake, whereas duodenal preloads have distinctively diminished satiating effects in OLETF compared to LETO rats. Our data compliment these results by revealing that gastric emptying of a nutrient load in OLETF and LETO rats show no apparent differences, regardless of amount of chow consumed, present in the stomach, or administered in liquid or solid phase. Gastric emptying of a nutrient load is distinguished by a period of increased emptying rate during periods of gastric fill, such as within a meal or resultant from gastric nutrient infusion, while emptying subsequent to meal termination is maintained at a slower, constant tempo (Kaplan et al. 1992). While this work does not address potential deficits in initial rates of emptying, our results show clearly that gastric emptying in OLETF rats after meal termination is unchanged relative to LETO rats.

Isolating the volumetric component of distension via intragastric balloon inflation allowed us to examine feeding responsiveness to a fixed volume of gastric distension in OLETF and LETO rats. Our results showed clear differences in intake patterns within OLETF and LETO rats during periods of gastric distension. Specifically, OLETF rats showed no response to 5 ml distension at age1 and reduced intake at only one time point during distension at age2, while LETO rats reduced intake from baseline at multiple time points during 5ml distension at both ages. When distended with a 10 ml gastric volume, responses were similar in OLETF and LETO rats at age1, however the magnitude of intake attenuation in response to distension appears to be greater in LETO relative to OLETF rats. Likewise, at age2, effects of 10 ml distension persisted for a longer period

in LETO than OLETF rats. These results suggest that OLETF rats have decreased sensitivity to gastric volumes during feeding, and thus may require a relatively greater degree of volumetric distension in order to reduce food intake compared to LETO rats. This observation parallels our recent finding showing that OLETF rats exhibit diminished responsiveness to intestinal nutrient infusion in a similar sham feeding design (De Jonghe et al. 2005). In this context, prior reports have identified the vagal signal induced by stomach distension to be largely a function of mechano-specific receptors (Mathis et al. 1998), in contrast to duodenal vagal afferents which respond to both mechano-, and chemo-sensation (Schwartz et al. 1995). In general, of the two main classes of vagal afferent endings, the intraganglionic laminar endings (IGLEs) and intramuscular arrays (IMAs), IMAs have been shown to primarily mediate signals of stretch and length change within the stomach, while IGLEs function more in response to more direct muscular contraction (Phillips and Powley 2000). Of particular relevance to our findings are the recent reports that knock-out animals lacking the Neurotrophin-4 (NT-4) gene exhibit deficient intestinal IGLE innervation which result in short term satiation deficits (Fox et al. 2001). In contrast, NT-4 knock-in mutants have been shown to be hypersensitive to CCK-induced satiation mediated through CCK-1 receptor activation (Chi and Powley 2003). Thus, it is possible that diminished IGLE responsiveness due to a congenital lack of the CCK-1 receptor in the OLETF rat may contribute to increased food intake in these animals. Given the current sham feeding design, however, it is unlikely that duodenal IGLEs play a significant role as no intestinal nutrient feedback was elicited. Nonetheless, when considering the functional differentiations in gastric and duodenal vagal innervation in an intact animal, one possible explanation for our sham feeding results is that gastric

vagal mechano-sensitivity is diminished in the OLETF rat, which would explain an attenuated decrease in intake due to stomach distension. Alternatively, a simpler explanation may be that the same degree of balloon inflation may not translate to the same degree of distension detection, due to increased stomach size in the OLETF rat. However, our gastric emptying results would not support the latter explanation because both small and large amounts of gastric nutrient volumes did not produce variable gastric emptying in any of our experiments at either age tested.

The suppressive effects of gastric distension on food intake have been shown to be largely mediated by activation of both gastric and hepatic branches of the vagus nerve [see for review (Powley and Phillips 2004)]. Our final experiment addressed whether diminished feeding response to gastric distension in OLETF rats could be explained, at least partially, through decreased vagal activation of distension signals. Gastric distension induces Fos within the NTS region of the DVC (Liu et al. 2004; van de Wall et al. 2005), an area established as the primary termination site for vagal afferent input from the stomach (Willing and Berthoud 1997). Furthermore, distension-induced Fos expression in the NTS is abolished via vagotomy (Mazda et al. 2004; Molinari et al. 2006). The current results of decreased DVC Fos expression within the NTS suggest that OLETF rats exhibit diminished vagal signaling induced by gastric distension relative to control LETO rats. This is in agreement with previous data from our laboratory (Covasa and Ritter 2005) as well as others (Glatzle et al. 2001) showing a decreased Fos expression in the enteric plexus, nodose ganglia and hindbrain of OLETF compared to LETO rats. In addition, levels of Fos neurons in LETO rats are comparable to Fos counts observed

following by other laboratories using a similar degree of gastric distension (Sabbatini et al. 2004).

Although CCK-1 receptors have been shown to participate in vagal responses following stomach distention, the precise degree of this participation has not been clear. Recently, van De Wall et al (van de Wall et al. 2005) reported that lorglumide did not diminish expression of Fos induced by 2 ml distension; however, it completely reversed the enhancement of distension-induced Fos expression by CCK. This suggests that lorglumide specifically reduces the response of vagal afferents to distension. The contributions by specific vagal afferent fiber populations in mediating gastric distension effects may vary according to the distension volume. It is possible that the relatively large 8 ml volume of gastric distension used in our study may result in involvement of CCK-1 receptor activity not captured when testing smaller distension volumes. Alternatively, the effect may be independent of CCK-1 receptors, and due to a direct effect of reduced vagal transmission of distension signals. A detailed analysis of multiple levels of gastric distension in the OLETF rat would be necessary to test this hypothesis. It is also worth mentioning that our Fos results do not indicate whether the observed decreased neuronal response is a contributor, or an artifact, of spontaneously increased meal size in the OLETF rat. Analysis of gastric distention-induced Fos expression using OLETF rats previously pair-fed to LETO controls in order to limit meal size may be useful in answering this question.

Recent work by Reidelberger et al. have provided interesting data focusing on peripheral vs. central feeding effects of blood brain barrier -permeable and non-permeable CCK-1 receptor antagonists (Reidelberger et al. 2004). From these data, it

appears likely that blockade of central or vagal CCK-1 receptors may be involved in separate, co-operative processes that lead to an overall increase in food intake in non-mutant animals. Indeed, the current Fos results would support peripherally associated CCK-1 receptor deficits in distension signaling. However, there are also reports that cells expressing gastric distension-induced Fos in the NTS activate projections that extend beyond the hindbrain, to forebrain structures such the paraventricular and supraoptic nuclei of the hypothalamus (Mazda et al. 2004). In this context, alterations in neuropeptide Y signaling within the dorsomedial and arcuate nucleus of the hypothalamus have been previously implicated in hyperphagia in the OLETF rat (Bi et al. 2001). Thus, it is possible that beyond vagal deficits described here, select hypothalamic nuclei implicated in gastric distension controls may be an additional contributor to aberrant feeding behavior in the OLETF rat.

It may also be argued that our findings of slightly decreased NTS Fos expression in sham treated OLETF compared to LETO rats may be indicative of a decreased responsiveness in general and not limited to gastric volume stimuli. This explanation is unlikely for two reasons. First, the actual raw total NTS Fos counts for the sham treatment is between 10 and 20 fold less than in distension treatments. While statistically different between strains, the small number of Fos positive nuclei is rather indicative of a slightly lower background in OLETF rats. This may be attributed to a lower activity of OLETF rats during testing as these animals have been shown to be hypoactive (Li et al. 2002). Second, the noted differential strain effects under gastric distension are of such exceedingly high magnitude relative to sham conditions, that any slight baseline differences of a few Fos counts under sham conditions have little impact on the

significance of distension effects between strains. This suggests that we did indeed observe specific differential effects of NTS Fos expression attributed to the gastric distension treatment, and not a generalized lower degree of Fos expression in OLETF compared to LETO rats.

To summarize, our findings reveal that OLETF rats, despite showing increased food intake, do not express deficits in their ability to control gastric emptying across multiple levels of gastric capacitance. Nevertheless, OLETF rats do show diminished feeding responses and neuronal activation induced by gastric distension. Thus, it is unlikely that hyperphagia in these animals involves deficient gastric emptying; however, decreased gastric mechanosensation and detection of gastric volume, in combination with previously described satiation defects, may facilitate overconsumption. The present findings seem to be discordant with work using acute CCK-1 receptor antagonism, and may suggest gastric and neuronal alterations in the OLETF not directly specific to CCK-1 receptor deficiency as possible mechanisms.

CHAPTER 3

INCREASED ORAL AND DECREASED INTESTINAL SENSITIVITY TO SUCROSE IN OBESE, PRE-DIABETIC OLETF RATS

INTRODUCTION

Animals (Sclafani 1987; Lucas and Sclafani 1990; Sclafani et al. 1996) as well as humans (Cross et al. 1994; Glanz et al. 1998; Birch 1999; Kant 2000; McCrory et al. 2002; Rolls 2003) overconsume when presented with highly preferred, palatable foods, that are either high in fat or carbohydrate, or both. For example, continuous access to sucrose solutions can promote or exacerbate hyperphagia in rats (Sclafani 1988; Sclafani et al. 1996) leading to increases in body weight (Sclafani 1987; Ackroff and Sclafani 1988; Lucas and Sclafani 1990; Sclafani et al. 1996). Likewise, consumption of such foods in large portions has been implicated as a major cause for the weight gain contributing to the increasing trends of obesity in the United States (Rolls 2003; Young and Nestle 2003).

Several recent data provide evidence that deficits in peripheral signaling mechanisms may account for the excessive meal-size and food intake observed in OLETF rats (Moran et al. 1998; Schwartz et al. 1999; Covasa and Ritter 2001; Bi and Moran 2002). However, our studies with CCK receptor antagonists in OLETF and LETO rats suggest that not all satiation deficits and hyperphagic behaviors in OLETF rats are directly related to the lack of CCK-1 receptors (Covasa and Ritter, 2001). Furthermore, Bi and Moran have recently shown altered central Neuropeptide Y (NPY) signaling within the dorsomedial hypothalamus as another contributor to hyperphagia in these animals in a

variety of real feeding paradigms (Bi et al. 2001; Bi and Moran 2002; Bi and Moran 2003; Bi et al. 2003; Bi et al. 2004). In contrast, there are scant data examining altered orosensory functioning in OLETF rats as a possible cause for hyperphagia and subsequent weight gain even though it is well established that orosensory components of the food, independent of post-ingestive effects, can have a significant impact on the amount of food consumed. In fact, rats will sham feed sucrose in a concentration dependant manner, when no post-absorptive effects are elicited (Weingarten and Watson 1982; Geary and Smith 1985; Nissenbaum and Sclafani 1987).

On this basis, the goal of the present study was twofold: 1) to determine the preference of prediabetic OLETF rats for sucrose solutions of various concentrations compared to their age-matched (6-8 wks), control LETO rats; 2) to assess whether increased preference for sucrose in the OLETF rats is due to pre- or postingestive feedback mechanisms. Therefore, in a series of experiments we employed short-access, two-bottle preference tests to examine potential strain differences in sucrose preference functions between OLETF and LETO rats. To assess participation of orosensory as well as post-gastric effects of sucrose solutions, in a separate study, rats were allowed to sham feed in the absence or presence of intraduodenal sucrose infusion.

METHODS

Subjects:

Five-week old male OLETF and LETO rats were obtained as a generous gift of the Tokushima Research Institute, Otsuka Pharmaceutical, Tokushima, Japan. All animals were individually housed in mesh-floored, stainless-steel hanging cages in a temperature-controlled vivarium while maintained on a constant 12:12-hr light-dark cycle (lights on at 0600). Rats were handled daily for a minimum of one week prior to the onset of experimental procedures. Tap water and pelleted rat chow (Purina 5001) were available ad libitum during the acclimation period. All protocols used were approved by The Pennsylvania State University Institutional Animal Care and Use Committee.

Surgical Procedures

Gastric Fistula & Duodenal Catheter Implantation

Gastric fistulae were implanted according to procedures listed in the methods sections of chapter 2. For duodenal catheter implantation, one end of the catheter (0.025 in. ID, 0.047 in. OD, Dow Corning, Midland, MI) was inserted via the gastric fistula through the pylorus and advanced beyond the pyloric muscle. A small silicone nub, 6 cm from the intraduodenal end of the catheter was passed through the pylorus and sutured on either side to one area of the basolateral surface of the duodenum. The peritoneum and abdominal muscles were simultaneously closed with absorbable sutures post-implantation. Wound clips were employed to close the abdominal skin incision, which were removed 7 days after surgery. The free end of the catheter was occluded with a stainless steel wire, which was removed immediately prior to each experiment when the

catheter was coupled to a syringe pump (Harvard, PhD 2000) to begin intestinal infusions. Between infusion sessions, the plugged free end of the catheter was tucked inside the outer fistula lumen before the steel screw was fastened. Rats were allowed a minimum of one week to recover from the fistula and catheter implantation surgery.

Procedures and experiments

Experiment 1. Sucrose preference of OLETF and LETO rats using short access, two-bottle choice test

Twenty rats (10 OLETF and 10 LETO) with average body weights of 244.1 ± 4.4 g and 197.3 ± 3.6 g, respectively, were divided in two groups (n=5 per strain) matched for body weight within each strain. Rats received two of the following sucrose concentrations during the training and testing period: 0.1 M and 1.0 M sucrose (Group 1) and 0.03 M and 0.3 M sucrose (Group 2). Rats were then placed on a water regimen consisting of overnight deprivation before the first training day, and exclusive water access for 1 hour in the evenings (1800-1900h) thereafter, throughout the whole experiment. Pelleted chow was available *ad libitum* except during the two-bottle tests. Rats were familiarized with the drinking schedule by giving them concurrent access to two bottles both filled with distilled water in the mornings for 1h between 1000-1100h and in the afternoon between 1500-1600h for two days. Then, rats were familiarized with the sucrose solution by presenting the average concentration of the two sucrose solutions (0.55 M or 0.165 M, for group 1 and group 2, respectively) in one bottle, with distilled water in the other bottle for two consecutive days to reduce neophobia. To control for side-preference, the position of each cylinder was changed between days and counter-

balanced within groups on the same day. For the next eight days, the two-bottle tests were performed with the first concentration of sucrose vs. water in the morning and the second concentration within the pairing vs. water in the afternoon, alternating every other day. Rats that received 0.1 M or 1.0 M sucrose never received other concentrations (i.e. 0.03 M or 0.3 M for sucrose). Conversely, the other half of the rats received only either 0.03 M or 0.3 M solutions, and never 0.1 M or 1.0 M sucrose. Sucrose and water intake were measured to the nearest 0.5 ml.

Experiment 2. Sham feeding of sucrose solution in OLETF and LETO rats in the absence of intestinal feedback

Three days prior to experiments, naïve OLETF and LETO rats (n=6 per strain) weighing 284.3 ± 6.5 g and 209.3 ± 5.5 g were placed on a fluid deprivation schedule during which they were allowed 6-h water access each day from 1400-2000 hr. Pelleted food was continuously available in the home cages except during licking sessions. After habituation, rats were acclimated to the sham feeding procedure by presenting them with 0.03 M sucrose for 60 min over three consecutive days. During testing, and following a brief 2-hr fast, the stainless steel screws occluding the gastric fistulas were removed, and stomach contents were lavaged with warm tap water to ensure minimal gastric volume and distension upon start of sham feeding. Rats were placed into Plexiglas sham feeding boxes and presented with either 0.03 M (1.03% w/v), 0.3 M (10.2% w/v), or 1.0 M (34.2% w/v) sucrose three times each over nine consecutive days in a randomized fashion. Sham intake was measured to the nearest 0.1 ml every 5 min over a 60-min feeding period (1030-1130 hr). In all sham feeding tests gastric drainage was collected in plastic graduated cylinders placed beneath the cages and the volume recorded at

experiment termination. If the volume of fluid ingested was greater than the volume of gastric drainage, or if gastric drainage did not occur within 15 s of the start of sham feeding, the data from that subject were discarded on the basis that the gastric fistula was not properly placed or functioning (Yox and Ritter 1988).

Experiment 3. Sham intake of sucrose in OLETF and LETO rats following intraduodenal sucrose infusion

OLETF and LETO rats (n=6 per strain, weighing 331 ± 5.4 g and 268 ± 7.0 g, respectively) previously used in Experiment 2 were used in intestinal infusion experiments. Scheduled access to chow and water, as well as sham feeding protocols, were identical to those described in Experiment 2 except that the sham-feeding session was extended to 90 min. All duodenal catheters were flushed with 0.5 ml of 0.15 M saline 30 min prior to infusion to ensure patency. Rats were presented with a single concentration (0.3 M) of sucrose solution throughout the experiment so that the effects of infusions with various sucrose concentrations could be compared. During testing, and after the stomachs had been cleared of gastric contents, rats were placed in sham feeding cages as previously described. The free end of the intraduodenal catheter was connected to a motorized digital syringe pump (Harvard, PHD 2000) set to deliver at a rate of 0.4 ml/min. Rats were presented with burettes filled with 0.3 M sucrose solution, and intestinal infusion began 10min after sucrose presentation and continued for 20min according to the procedure employed by Greenberg et al. 1990 (Greenberg et al. 1990). Each rat received a total volume of 8.0 ml of infusate. After infusion ceased, rats were allowed to sham feed sucrose for an additional 60 min to detect changes in sham intakes

following sucrose infusion. Thus, rats had access to 0.3 M sucrose for a total of 90 min. Before sucrose infusion sessions began, two days of 0.15 M saline infusions were performed to establish a stable baseline of 90 min sham intake. Rats then received each of three concentrations of sucrose infusions: 0.3 M (0.83 g/8 ml), 0.6 M (1.6 g/ 8 ml), and 1.0 M (2.7 g/8 ml) followed by another 0.15 M saline baseline infusion test. Rats were tested twice under each sucrose concentration. The patency of the duodenal catheters was assessed by adding red food dye to all infusates. If red coloring appeared in the gastric drainage, data for that animal were not included in the analysis.

Oral Glucose Tolerance Test (OGTT)

Two days following experimental testing, an oral glucose tolerance test was performed in a subset of 8 rats (n=4 per strain, 12 wks of age, weighing 342 ± 6.41 g and 274 ± 6.8 g for OLETF and LETO, respectively) within each of the two experimental groups to determine whether or not OLETF rats had developed NIDDM. After a 16hr fast, an oral glucose load (2 g/kg) was delivered to each rat via oral gavage. Blood glucose was measured before gavage and 30, 60, 90, and 120 min post-glucose administration via glucometer (LifeScan, One-Touch Basic). Animals were classified as diabetic if the peak level of plasma glucose was ≥16.8 mM and a peak glucose level at 120 min > 11.2 mM (Kawano et al. 1992).

Statistical Analysis

For Experiment 1, two-way repeated measures analysis of variance (rmANOVA) performed with strain and sucrose concentration as main effects. Total intakes of both solutions within the pairing (sucrose + water) were used to calculate preference

percentages according to the following formula: Preference percentage = [volume of sucrose solution (ml) x 100/ total volume of sucrose and water (ml)].

Sixty-minute sham intake in Experiment 2 was analyzed by appropriate two-way rmANOVA with strain and sucrose concentration as main factors. One-way rmANOVA was performed to test concentration effects of sucrose within OLETF or LETO rats. ANOVA results were subsequently analyzed by Tukey's honestly significant difference (HSD) test post-hoc tests when applicable for both experiment 1 and 2.

For Experiment 3, one way rmANOVA was used to calculate all individual 5-min intake bins as well as cumulative 5 min intakes in both OLETF and LETO rats within each infusion groups. Percent suppression of 0.3 M sucrose sham intake over the 90 min session was calculated using a two-way rmANOVA with strain and infusion concentration as main effects. Percent suppression was calculated using the following formula: Percent Suppression = 1-(experimental/ baseline) X 100. One-way rmANOVA was calculated to test concentration effects of sucrose infusion on sham intake within OLETF or LETO rats. These results were again analyzed post-hoc by Tukey's HSD when necessary.

Planned t-tests were calculated to compare blood glucose levels in OLETF and LETO rats following an OGTT. All data are expressed as means $\pm$ SEM. Differences were considered statistically significant if $P<0.05$. All statistical analyses were carried out with PC-SAS (version 8.02, SAS Institute, Carey, NC).

RESULTS

Experiment 1: Sucrose intake following short access, two-bottle tests in age-matched OLETF and LETO rats

There were no overall within strain differences in sucrose intake in the morning versus afternoon sessions. Therefore, data were pooled for the subsequent analyses. Pre-diabetic OLETF rats exhibited a greater preference for 0.3 M (91.2 ± 1.7 % and 78.5 ± 3.4 % for OLETF and LETO, respectively; $F(1,8)=11.4$; $P<0.01$) and 1.0 M sucrose (65.3 ± 1.2 % and 57.5 ± 2.7 % for OLETF and LETO, respectively; $F(1,8)=6.66$; $P<0.05$), than LETO controls in a 60 min two-bottle choice test [Fig. 3.1]. OLETF rats consumed a greater absolute volume of 0.3 M (34.6 ± 2.9 ml and 21.5 ± 1.5 ml for OLETF and LETO, respectively; $F(1,8)=16.5$; $P<0.01$) and 1.0 M (22.1 ± 1.6 ml and 12.9 ± 0.6 ml for OLETF and LETO, respectively; $F(1,8)=29.8$; $P<0.01$) sucrose, while paired water intakes during these sessions did not differ significantly (P=ns). No strain differences in intake or preference were noted for 0.03 M and 0.1 M sucrose consumption, as well as for the respective paired water intakes (P=ns). Average daily caloric intake (chow + sucrose) was significantly higher [$F(1,16)= 22.30$; $P<0.01$] in OLETF compared to LETO rats (89.2 ± 4.6 and 70.0 ± 3.1 kcal/day, respectively).

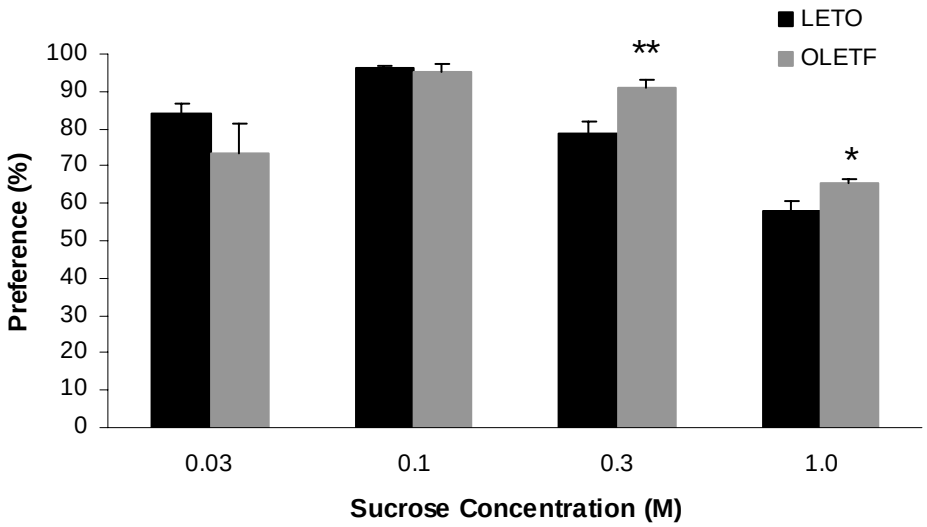


Figure 3.1: Sucrose intake following short access, two-bottle tests in OLETF and LETO rats. OLETF rats preferred 0.3 M and 1.0 M sucrose to a greater extent than LETO controls in a 1hr two-bottle choice test. Lower concentrations (0.03 M, 0.1 M) were preferred equally between groups (*P<0.05,**P<0.01 between strains).

Experiment 2: Sucrose sham feeding in OLETF and LETO rats

For both OLETF and LETO rats, sham intake increased as a function of concentration [F(2,13)=44.9; P<0.0001 and F(2,15)=7.48; P<0.01 for OLETF and LETO rats, respectively]. However, OLETF rats drank significantly less 0.03 M sucrose [33.8 ± 4.8 ml and 58.3 ± 7.3 ml for OLETF and LETO, respectively; F(1,9)=7.1; P<0.05] than LETO controls during a 60 min sham-feeding session [Fig 3.2]. In contrast, OLETF rats sham fed significantly greater amounts of 1.0 M sucrose [109.9 ± 6.5 ml and 81.0 ± 3.9 ml for OLETF and LETO, respectively; F(1,9)=15.9; P<0.01] than LETO animals. Both strains consumed a similar amount of 0.3 M sucrose during 60 min sham feeding (P=ns). Post hoc testing revealed that while OLETF rats increased consumption across all three concentrations administered (P<0.01 and P<0.001 for 0.03 M to 0.3 M sucrose, and 0.3 M to 1.0 M sucrose, respectively), LETO rats only increased intake of 0.03 M to 0.3 M sucrose (P<0.05), and did not increase intake of 0.3 M to 1.0 M sucrose (P=ns).

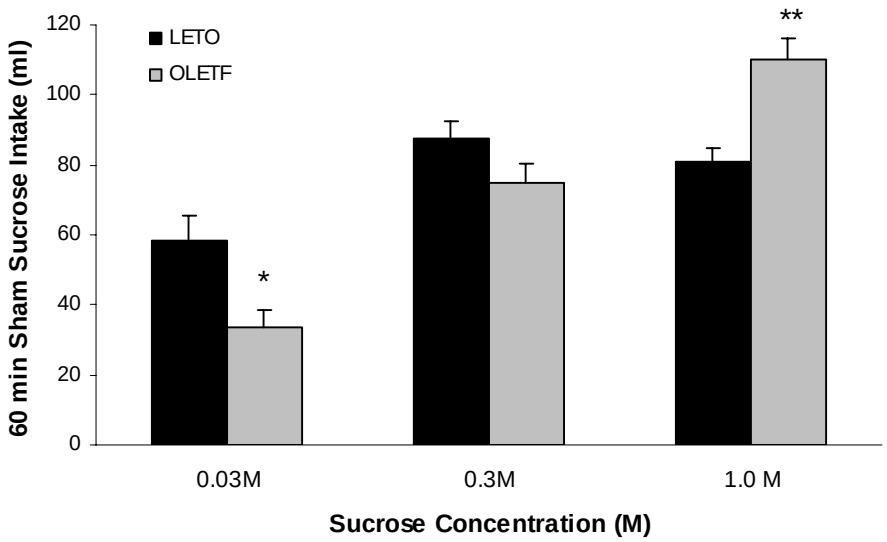


Figure 3.2 Sham feeding of three concentrations of sucrose (0.03 M, 0.3 M and 1.0 M) in OLETF and LETO rats. Sham fed OLETF rats consumed significantly more 1.0 M sucrose and less 0.03 M sucrose than LETO rats over a 60-min period. There was no noted strain difference for 0.3 M sucrose. (*P<0.05,**P<0.01 between strains).

Experiment 3: Sham-feeding in OLETF and LETO rats following intestinal infusion of sucrose

Intraduodenal infusion 0.15 M saline did not lead to significantly different 90 min sham intake of 0.3 M sucrose between OLETF and LETO rats (P=0.138). Figure 3B shows that no overall strain difference in sham intake was noted in response to 0.3 M sucrose infusion (P=ns). OLETF rats, however, exhibited an initially higher (P<0.05) sham intake of 0.3 M sucrose than LETO controls, which was attenuated during infusion of 0.3 M sucrose [Fig. 3.3A]. Cumulative sham intake was significantly enhanced (P<0.05) until 50 min post-infusion [Fig. 3.3B].

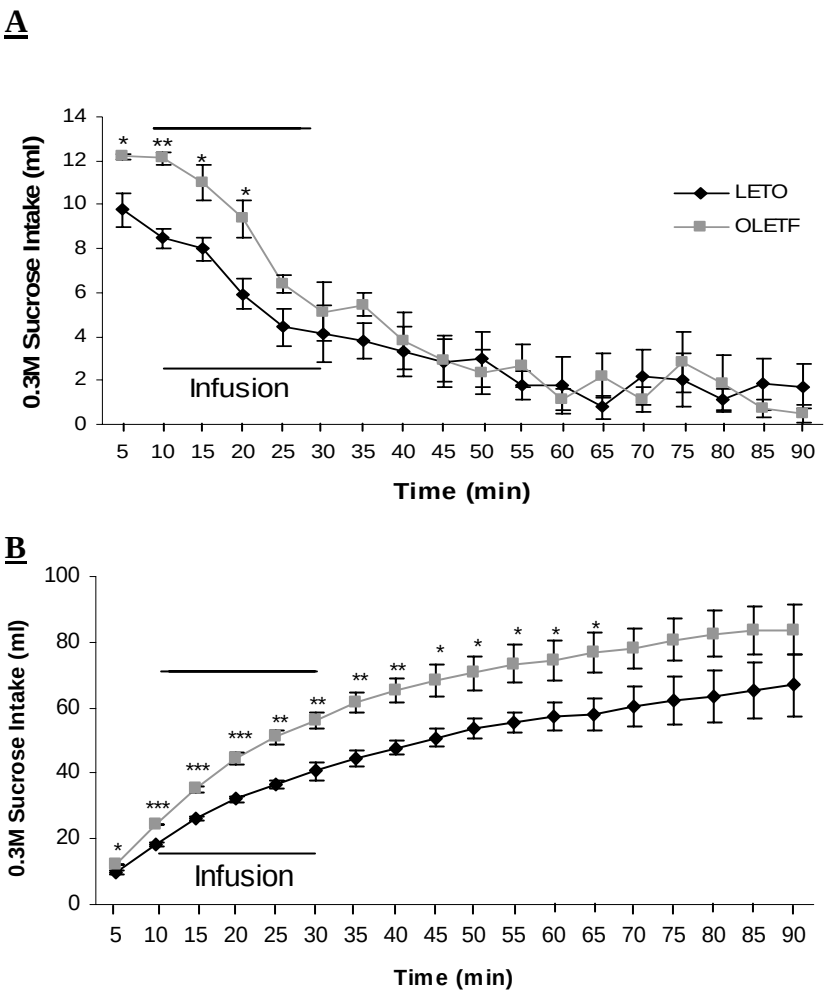


Figure 3.3 Ninety-minute sham intake of 0.3 M sucrose in response to 20-min duodenal infusion of 0.3 M sucrose. (**A**) OLETF rats had an initial higher sham intake of 0.3 M sucrose than LETO controls before the start of the infusion. (**B**) Cumulative sham intake data show that OLETF rats had significantly higher sham intakes compared to LETO throughout the 70-min sham feeding. However, no overall strain difference in sham intake was noted at 90 min. (*P<0.05,**P<0.01 between strains).

Infusion of 0.6 M sucrose resulted in significantly higher sham intakes in OLETF versus LETO rats (P<0.05) [Fig. 3.4B]. Intakes before infusion of 0.6 M and 1.0 M sucrose were again significantly higher (P<0.05 and P<0.01 for 0.6 M and 1.0 M sucrose infusion, respectively) for OLETF rats. In contrast to results from saline and 0.3 M sucrose infusions, however, intraduodenal infusion of 0.6 M and 1.0 M sucrose in OLETF rats showed marked differences in sham intake compared to LETO controls following infusion termination. Figure 3.4A shows that while both strains decrease their

sham intake in response to infusion of 0.6 M sucrose, only OLETF rats show an immediate increase in 5-min sucrose intake directly after infusion end. This increased intake remained significantly elevated ($P<0.05$) relative to LETO controls for each 5-min recording until 30 min post-infusion. Sham intake at each 5-min time point recorded after infusion onset was significantly higher ($P<0.05$) in OLETF rats compared to LETO rats [Fig. 3.4B].

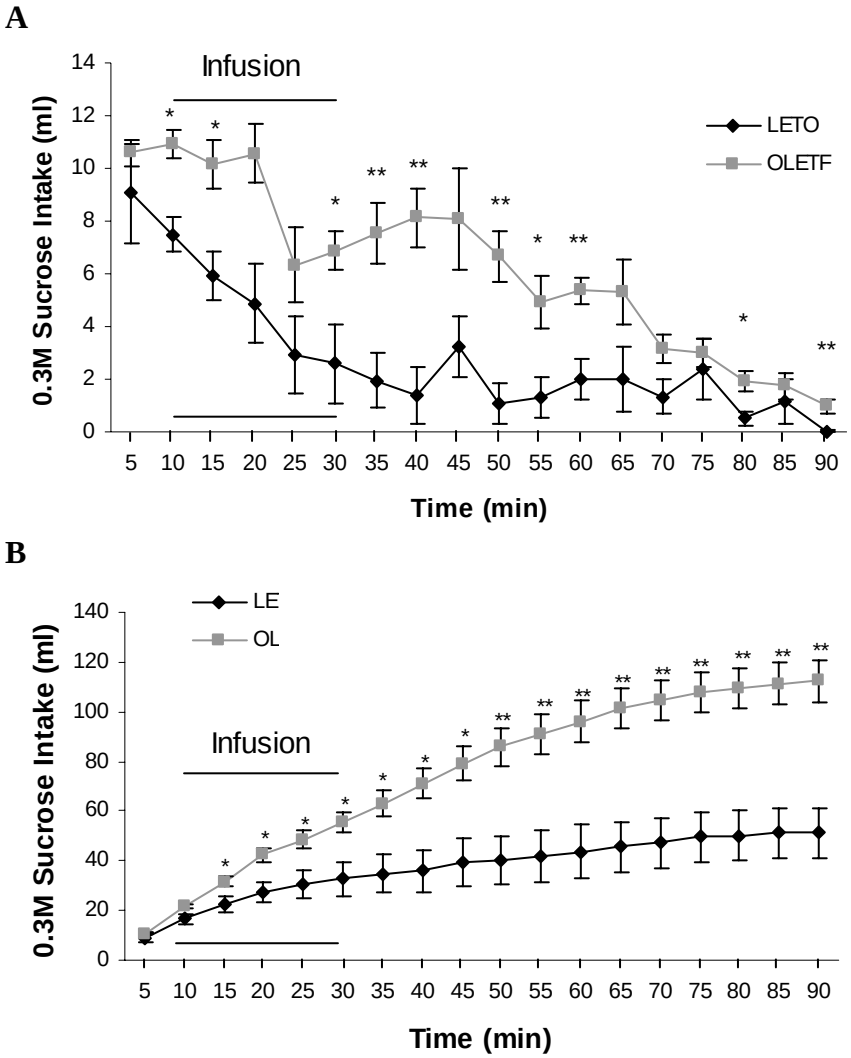


Figure 3.4 Ninety-minute sham intake of 0.3 M sucrose in response to 20-min duodenal infusion of 0.6 M sucrose. (**A**) OLETF rats show an immediate increase in 5-min sucrose intake after the end of 0.6 M sucrose infusion, in contrast to LETO controls which maintain their lower level of sham intake. This increased intake remained elevated relative to LETO controls for 30-min post-infusion. (**B**) Cumulative sham intakes post-infusion onset of 0.6 M sucrose were significantly higher in OLETF compared to LETO rats. (*$P<0.05$,**$P<0.01$ between strains).

Figure 3.5B illustrates that OLETF rats had significantly higher ($P<0.05$) overall intakes of sucrose compared to LETO rats when intraduodenally infused with 1.0 M sucrose. Infusion of 1.0 M sucrose again decreased 5-min intakes in both strains; however, LETO rats consumed almost no sucrose after infusion ceased [Fig 3.5A]. Conversely, OLETF rats increased their sham intake following infusion termination, reaching significantly higher levels [$F(1,8)=8.02$; $P<0.05$] than LETO controls at 25 min post-infusion. Sham intake at all time points in the 90 min sham session were significantly higher ($P<0.05$) in OLETF rats compared to LETO animals.

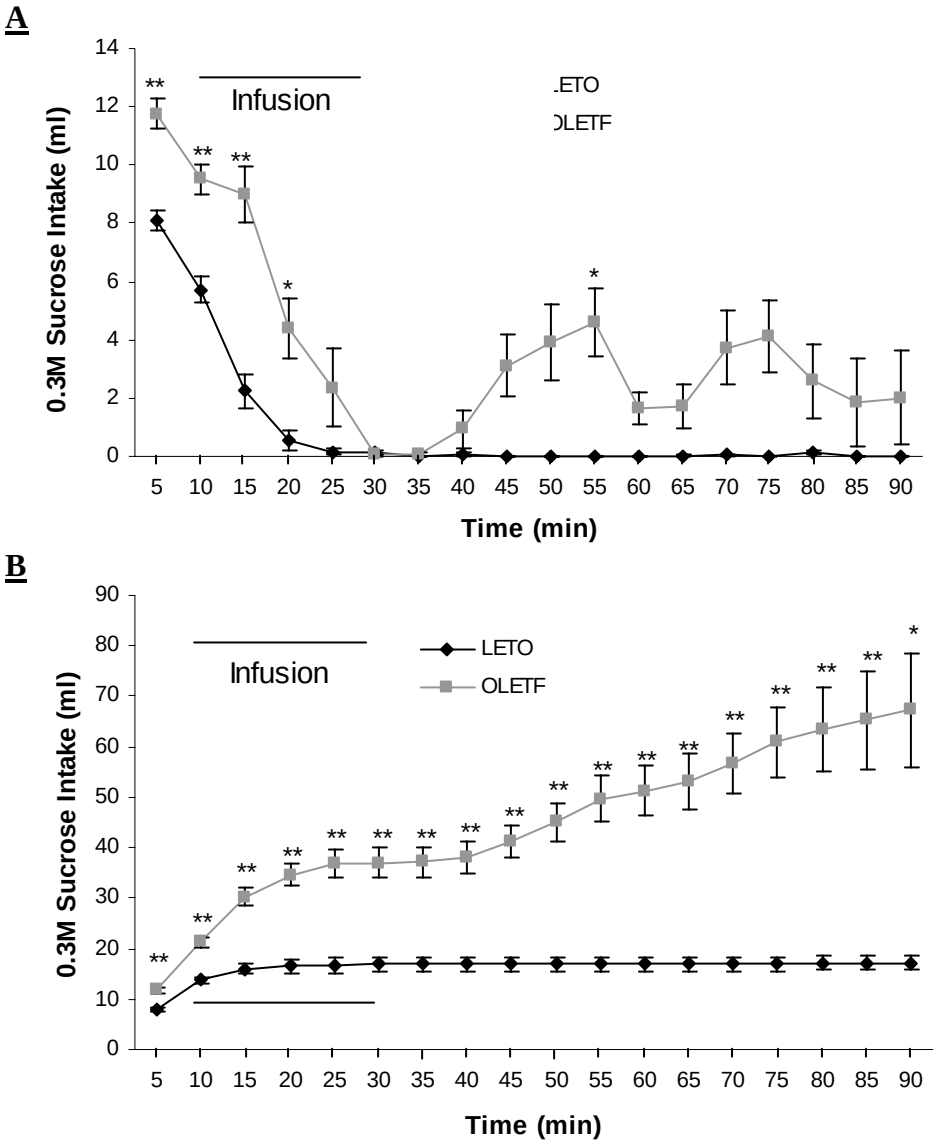


Figure 3.5 Ninety-minute sham intake of 0.3 M sucrose in response to 20-min duodenal infusion of 1.0 M sucrose. (**A**) Infusion of 1.0 M sucrose decreased 5-min intakes in both strains; however LETO rats consumed almost no sucrose after infusion ceased. OLETF rats significantly increased their sham intake after infusion ended in comparison to LETO controls. (**B**) Cumulative sham intake at all time points in the 90-min sham session were significantly higher in OLETF rats compared to LETO animals. (*P<0.05,**P<0.01 between strains).

For both strains, suppression of 0.3 M sucrose sham intake increased as a function of infusate concentration [$F(2,10)=55.6$; $P<0.0001$ and $F(2,11)=6.33$; $P<0.05$ for OLETF and LETO rats, respectively]. Figure 6 shows that infusions of 0.6 M and 1.0 M sucrose produced significantly enhanced suppression of 0.3 M sucrose sham intake in LETO rats

relative to OLETF rats [F (1,8)=18.10; P<0.01 and F(1,8)=11.04;P<0.05 for 0.6 M and 1.0 M infusions, respectively]. OLETF and LETO rats show no differential responsiveness to infusion of 0.3 M sucrose (P=ns). Post hoc tests revealed that LETO rats suppressed intake significantly across all three concentrations of infusate (P<0.001 for both 0.3 M to 0.6 M, and 0.6 M to 1.0 M sucrose). In contrast, suppression of sham intake in OLETF rats was enhanced only between 0.6 M to 1.0 M sucrose infusion (P<0.05), while no difference in suppression was noted between 0.3 M and 0.6 M sucrose infusion (P=ns).

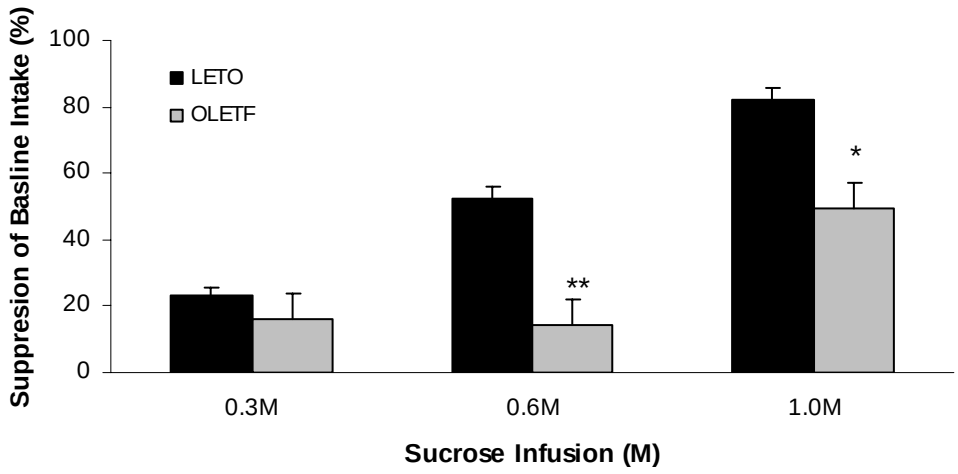


Figure 3.6 Percent Suppression of 0.3 M sham sucrose intake in response to intraduodenal infusion of sucrose. OLETF and LETO rats show no differential responsiveness to infusion of 0.3 M sucrose. Infusions of 0.6M and 1.0 M sucrose, however, produced enhanced suppression of 0.3 M sucrose sham intake in LETO rats relative to OLETF rats. (*P<0.05,**P<0.01 for strain differences in percent suppression)

Oral Glucose Tolerance Test (OGTT)

Results of the OGTT performed at the end of the study on samples of rats from each experimental group showed that 12 wk-old OLETF rats were pre-diabetic since they did not show elevated blood glucose levels indicative of NIDDM [Fig. 7]. However, OLETF rats showed significantly higher [F(1,6)=42.2; P<0.001] blood glucose peaks at

30 min relative to LETO rats. At all other time points, blood glucose in OLETF and LETO rats did not differ significantly (P=ns).

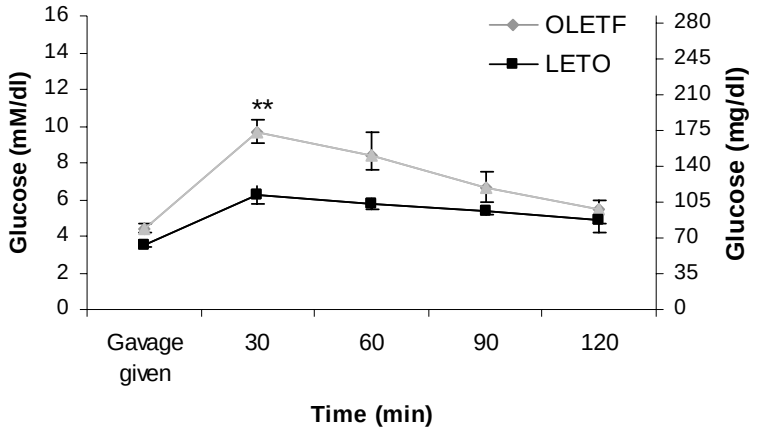


Figure 3.7 Oral Glucose Tolerance tests in 12-wk old OLETF and LETO rats following study completion. OLETF rats have substantially higher blood glucose peaks at 30 min relative to LETO controls. At all other time points, blood glucose in OLETF and LETO rats did not differ significantly. (**P<0.001 between strains).

DISCUSSION

These results show that in short access, two-bottle preference tests, prediabetic OLETF rats exhibit a markedly enhanced preference for sucrose compared to non-mutant LETO rats. We also show that OLETF rats consumed more sucrose than LETO rats in the absence of gastric or intestinal feedback stimulation. Finally, OLETF rats were less responsive to the inhibitory effects of intraduodenal infusion of sucrose compared to LETO rats. To our knowledge, these are the first studies to demonstrate an altered preference for sucrose in the CCK-1 receptor deficient rat using two different paradigms: the short access, two bottle test and sham feeding preparation.

Intake and preference for sucrose in rats have been shown to depend on solution concentration (Davis 1973). In our short access test, OLETF as well as LETO rats

displayed an equal preference for sucrose solutions at the lower concentration range (0.03, 0.1 M). As sucrose concentration increased beyond 0.1 M, the overall preference decreased. However, OLETF rats had an increased preference for sucrose solutions of the two highest concentrations (0.03 M and 1.0 M). In other words, OLETF rats display a right-shifted sucrose preference curve relative to LETO rats.

It is well established that both orosensory and postingestive components of a food can modulate intake (Greenberg et al. 1990; Holder 1991; Davis et al. 1995; Sclafani et al. 1999). Given that sucrose is both a toothsome stimulus and also contains calories, data from the first experiment did not allow us to interpret the relative contribution of orosensory and post-gastric effects of sucrose on preference. Prior work has focused primarily on gastric and post-gastric mechanisms mediating regulatory deficits in CCK-1 receptor rats (Moran et al. 1998; Schwartz et al. 1999; Covasa and Ritter 2001). The sham feeding preparation allowed us to test participation of orosensory components of sucrose in the absence of any appreciable gastric and post-gastric stimulation (Weingarten and Watson 1982). In this paradigm, sucrose ingested empties from the stomach via a drainage tube providing minimal contact of the solution with the gastric mucosa (Sclafani and Nissenbaum 1985).

We observed that OLETF rats naïve to the post-gastric effects of sucrose showed an increased sham intake of the highest sucrose concentration presented (1.0 M), and a decreased intake of the lowest concentration (0.03 M), relative to LETO control animals. These data are in concert with results from the two-bottle preference testing in that strain differences occurred primarily at relatively higher concentrations of sucrose, that is OLETF rats exhibit an unaltered concentration discrimination function with a right-

shifted preference function. These observed changes in sucrose intake are suggestive of alterations in the motivational modulation of taste functioning in OLETF rats, rather than diminished responsiveness to the primary gustatory signal.

The mechanisms by which OLETF rats increase their intake and preference for sucrose are not known, however the absence of CCK-1 receptors is one likely candidate. Numerous studies have shown that exogenous CCK administration decreases sham intake (Gibbs et al. 1973; Kraly et al. 1978; Falasco et al. 1979; Forsyth et al. 1985) while blockade of the CCK-1 receptor with a specific antagonist potently inhibits or abolishes this diminution (Brenner and Ritter 1995; Brenner and Ritter 1998). Administration of CCK-1 receptor antagonists alone, however, has not been shown to alter sham intake, suggesting that activation of the CCK-1 receptor by endogenous levels of CCK does not play a regulatory role in sham feeding (Reidelberger et al. 2003). Furthermore, data examining the effects of CCK on the orosensory components of palatable foods have not provided conclusive evidence supporting a role for CCK-1 receptors in the orosensory control of food intake (Waldbillig and Bartness 1982; Gosnell and Hsiao 1984; Eckel and Ossenkopp 1994). Lastly, administration of a CCK-1 receptor antagonist does not alter conditioned flavor preferences in intact rats (Perez et al. 1998). Taken together, it appears that increased sham intake in the OLETF rat, lacking the functional CCK-1 receptor, is not due to a primary deficit in CCK-1 receptor activation.

Our data also show that sham fed OLETF rats are less responsive to intestinal infusion of sucrose than LETO controls. In addition, the kinetics of sham intake in OLETF rats revealed an initially increased rate of sucrose consumption when post-gastric effects of sucrose were not yet present, as well as increased consumption following

infusion of the two highest concentrations of sucrose infusate. Such discrepancies in intake between strains both pre- and post-infusion appear to additively magnify increased sham intake in OLETF rats. These latter findings are in agreement with prior demonstrations in OLETF rats that illustrate reduced sensitivity to gastric and intestinal preload nutrient infusions in real feeding tests (Schwartz et al. 1999; Covasa and Ritter 2001).

There is ample evidence attesting the role of CCK-1 receptors in nutrient induced suppression of food intake (Ritter 2004). For example, CCK-1 receptor antagonists have been shown to attenuate suppression of both real and sham feeding following intraduodenal nutrient infusion (Yox et al. 1992; Reidelberger et al. 2003). Therefore, one mechanism by which OLETF rats are less responsive to the suppression of intake by sucrose infusion might be due to their lack of CCK-1 receptors. However, in addition to the well characterized CCK-1 receptor deficits, OLETF rats may have alterations in other feeding related signaling pathways, such as in those involved in food reward processes.

The intake of preferred foods has been shown to affect opioid, serotonergic and dopaminergic systems (Simansky 1996; Hoebel 1997; Kelley et al. 2002). When given intermittent access to sugar solutions, rats exhibit increased binding of D1/D5 dopamine receptors and μ-1 opioid receptors in the nucleus accumbens (NAcc) (Colantuoni et al. 2001). Conversely, opioid antagonists reduce intake of palatable foods in real (Cooper 1983; Cooper et al. 1985; Lynch 1986) and sham (Kirkham and Cooper 1988; Kirkham 1990) feeding, and decrease sucrose palatability in taste reactivity tests (Parker et al. 1992). While it seems that CCK-1 and opioid receptors do not interact to alter food intake (Pupovac and Anderson 2002), an enhanced sucrose preference in the OLETF rats may

also be due to deficits in opioid signaling in addition to those attributed to lack of CCK-1 receptor. However, this possibility has yet to be tested.

Serotonergic signaling may also play a role in sweet preference modulation in OLETF rats. Exogenous 5-HT administration has been shown to not only reduce intake, but also diminish preference of preferred saccharin and sucrose solutions in intact animals (Montgomery and Burton 1986). Furthermore, evidence for an interaction between the cholecystokinergic system on both central (Poeschla et al. 1992; Helm et al. 2003) and peripheral (Hayes et al. 2004; Hayes et al. 2004) serotonergic receptors in the control of food intake have been reported. The effects of serotonergic manipulations in the OLETF rat on food intake and preference, however, have not been addressed.

Finally, recent studies assessing dopamine functions in the OLETF rats provide indirect evidence that may shed some light on possible mechanisms of enhanced sucrose preference in the OLETF rats (Shilling and Feifel 2002; Feifel et al. 2003; Hajnal et al. 2004). Indeed, evidence supporting the role of central DA as a mediator of food reward in non-mutant animals has been postulated for some time (Hernandez and Hoebel 1988; Phillips et al. 1993). Sham feeding sucrose in non-mutant (Sprague-Dawley) male rats has been shown to trigger DA release as a function of sucrose concentration (Hajnal et al. 2004). Furthermore, intraperitoneal (IP) administration of the DA D2/D3 receptor antagonist raclopride reduced sham feeding of sucrose in a dose-dependent manner (Hsiao and Smith 1995). Administration of D1/D5 receptor blockers directly in the NAcc suppresses intake. Conversely, increasing DA levels provoke greater intakes and preference for sucrose solutions (Hajnal and Norgren 2001). Learned preference for sucrose has also recently been shown to be decreased with administration of dopamine

receptor blockers (Yu et al. 2000; Yu et al. 2000). Furthermore, CCK has been shown to stimulate central DA release via activation of CCK-1 receptors (Crawley 1991) .While there are only sporadic data to support the theory of an altered DA signaling additional to CCK signaling abnormalities in OLETF rats (Hajnal et al. 2004), it is plausible that such deficits could interfere with reward mechanisms and, in turn, contribute to the manifestation of altered preferences for palatable food stimuli.

In summary, OLETF rats prefer sucrose to a greater extent than non-mutant LETO control rats in a short access two-bottle choice test. When post-ingestive caloric and chemosensory properties of sucrose are removed via gastric fistula preparation, OLETF rats sham feed more of a relatively high concentration of sucrose than LETO rats. In the presence of intestinal feedback stimulation OLETF rats display diminished sensitivity to sucrose infusion as evidenced by greater sham intakes of sucrose when compared to non-mutant LETO animals. The specific mechanisms behind these phenomena are not well defined; however it is likely that secondary anomalies beyond the lack of CCK-1 receptors, such as those involving the dopaminergic system may mediate the altered ingestion of sucrose solutions in OLETF rats.

CHAPTER 4

INCREASED REAL FEEDING AND CONDITIONED PREFERENCE FOR SWEET STIMULI IN OLETF RATS

INTRODUCTION

It is well known that amount consumed within a meal is a function of both oral and post-oral nutrient properties of foods (Sclafani and Ackroff 2004). Heightened orosensory responses to preferred tastants in OLETF rats (11, 20) suggest that altered oral or taste sensation may additionally compound hyperphagia attributed to known peripheral satiation defects in these animals. In this context, learned associations between the flavor of food and its post-ingestive consequences can influence food intake and preference (Holder 1991; Sclafani 1997). Compelling evidence exists that learned preferences can be mediated by associations of nutritive feedback. Indeed, rats can acquire preference from odors and tastes that have been paired with intragastric or intraduodenal infusions of sugars (Elizalde and Sclafani 1990; Azzara and Sclafani 1998), as well as when given prior access to an arbitrary cue flavor solution paired with a specific palatable food (Capaldi et al. 1983).

It has also been established that rats subjected to periods of acute food deprivation increase subsequent consumption of palatable foods (Smith and Duffy 1957; Collier and Bolles 1968). The exact mechanism responsible for this increase, however, is not entirely clear. For instance, food restricted rats can show heightened intake of both caloric sucrose and non-caloric saccharin solutions (Smith and Duffy 1957). It appears that an

increase in motivation to eat due to caloric deprivation does not comprise the sole impetus for increased consumption under restrictive conditions, but rather increases the overall responsiveness to gustatory properties of the food. Indeed, there are reports of enhanced hedonic response (Berridge 1991), as well as lower taste thresholds for sweet solutions under deprived conditions (Campbell 1958; Zverev 2004) that support this notion.

Bi and Moran have shown that in addition to overconsuming more chow over 24 hrs under *ad libitum*- fed conditions, OLETF rats, also overconsume chow to a higher degree than LETO rats following a 24 hr fast (Bi and Moran 2003). The effect of food restriction on the intake of preferred liquid solutions in these animals has not been tested. Therefore, the first aim of the present study was to test food deprivation effects on intake and preference for normally preferred sucrose solutions in OLETF and LETO rats.

Conditioning a flavor preference has been used to dissociate positive orosensory vs. post-ingestive effects in the consumption or preference for a particular food. Several studies have provided evidence that the formation and expression of flavor-taste and flavor-calorie associations are regulated differentially by levels of hunger (Capaldi et al. 1983; Fedorchak and Bolles 1987; Harris et al. 2000). For example, hungry rats show increased preference for a conditioned stimulus (CS) flavor associated with sucrose relative to sated animals. In contrast, there appears to be no differential effect of hunger on flavor preference for a flavor associate of saccharin (Fedorchak and Bolles 1987; Capaldi and Hunter 1994; Harris et al. 2000). Furthermore, more recent data suggest that food deprivation affects the expression, but not acquisition, of conditioned flavor preference (Yiin et al. 2005; Yiin et al. 2005).

We have previously shown that OLETF rats exhibit an increased intake as well as preference for palatable sucrose in both real and sham feeding paradigms (De Jonghe et al. 2005; Hajnal et al. 2005) . This increased sensitivity to oral stimulation by sweet tastes (De Jonghe et al. 2005; Hajnal et al. 2005) suggests that orosensory components of sucrose solutions impart enhanced contributions to sucrose intake relative to LETO controls. To investigate effects of food deprivation on learned preferences for sweet solutions, we employed a conditioned flavor-calorie procedure using palatable sucrose or saccharin solutions as unconditioned stimuli (US) and tested intake of paired conditioned stimulus (CS) flavors under both deprived and free-fed conditions. If OLETF rats are more sensitive to the orosensory properties of sweet solutions and less sensitive to the post-absorptive effects, we would expect these rats to be less responsive to potential differences in expression of conditioned sucrose flavor preference induced by acute food restriction relative to LETO rats, when tested in a conditioned sweet preference paradigm. We also hypothesized that if OLETF rats are indeed more sensitive to orosensory associations of sweet solutions, then saccharin should produce a greater conditioned flavor preference in OLETF rats compared to LETO rats when animals are. Therefore, our second aim was to assess possible alterations in the expression of conditioned flavor preferences for both caloric and non-caloric sweet solutions in OLETF and LETO rats under food deprived and *ad libitum* fed conditions.

METHODS

Subjects:

Male OLETF and LETO rats were obtained as a generous gift of the Tokushima Research Institute, Otsuka Pharmaceutical, Tokushima, Japan. All rats were housed individually in mesh-floored, stainless-steel hanging cages and maintained in a temperature-controlled vivarium on a constant 12:12-hr light-dark cycle (lights on at 0600). Animals were handled daily for a minimum of one week prior to the onset of experiments. Tap water and pelleted rat chow (Purina 5001) were available ad libitum throughout experiments, except where indicated otherwise. All protocols used were approved by The Pennsylvania State University Institutional Animal Care and Use Committee.

Intake and Preference of 0.3 M and 1.0 M sucrose in OLETF and LETO rats under ad libitum and food-deprived conditions

Twenty-four week old OLETF and LETO rats (n=5 per strain, weighing 537 ± 29.6 g and 435 ± 6.7 g, respectively) were used for the following experiments. In sucrose intake studies, all rats received daily, 1 hr access to calibrated glass drinking burettes containing 0.3 M sucrose between 1000 and 1100 hr everyday. Prior to daily sucrose presentation, 24 hr chow intake and spillage was measured. After the establishment of a stable baseline sucrose intake under *ad libitum* fed conditions (6-8 days), rats were food deprived across two levels of chow restriction. In the low restriction condition, animals were tested 24 hrs after presentation of a food ration limited to 75% baseline daily ad libitum chow intake. Given the well-documented differences in daily chow intake

between OLETF and LETO rats, the low restriction group was rationed in this manner in order to provide comparable degrees of a food restriction as a function of daily intake within strain. In the high restriction condition, rats had no access to food for 24 hrs prior to testing (i.e., both strains were deprived of 100% *ad libitum* daily chow intake). One hour daily sucrose intakes under either *ad libitum* or food restricted conditions were compared to test how differences in hunger motivation would affect intake of a palatable sucrose solution. Daily sucrose access time and duration remained unaltered throughout experimentation.

Food restriction conditions were imposed a minimum of 2 days apart, to both allow for compensatory chow intake, and to assess possible shifting in non-deprived sucrose baseline intakes as a result of intermittent access. Every deprivation condition was repeated a minimum of two occasions. This study was first completed using 0.3 M sucrose as test solution, followed by an identical experiment using a more concentrated 1.0 M sucrose solution in the same groups of rats. Sucrose intake was measured to the nearest 0.1 ml.

In a further experiment, using the same deprivation schedule and sucrose access periods, daily 1 hr two-bottle choice tests were performed using 0.3 M versus 1.0 M sucrose to assess sucrose concentration preference changes as a function of motivational state. To control for side-preference, the position of each sucrose burette was alternated daily and counter-balanced within animals on the same testing day.

Conditioned preference for caloric and non-caloric sweet solutions in food deprived or non-deprived OLETF and LETO rats

Twenty, 10 wk old,, naïve 10 wk old pre-diabetic OLETF and LETO rats were divided in two groups (n=5 per strain, weighing 287.2 ± 6.8 g and 220.4 ± 3.8 g, respectively). A two-day habituation period preceded experimentation during which all animals were given brief access to 3 ml of an unflavored 0.2% saccharin solution at 1000 hr to familiarize the animals with one bottle acceptance. As shown in Figure 4.1, over the next eight calendar days all rats were exposed to a 3 ml volume of a cue flavored solution (conditioned stimulus, CS) of either grape or cherry flavored saccharin (0.2% saccharin (w/v), and 0.05% Kool Aid (w/v)) immediately preceding 30 min presentation of an assigned unconditioned stimulus (US) solution. One group of OLETF and LETO rats received 3 ml grape saccharin (CS+) paired with 30 min ad libitum exposure to 0.3 M unflavored sucrose (US+) and 3 ml cherry flavored saccharin (CS-) with 30 min access to plain water (US-), while an additional group of rats were conditioned using the opposite CS-US pairings. All animals were given CS+/US+ and CS-/US- pairings on alternate calendar days for a total of 4 exposures to each pairing prior to CS preference testing. All solutions were presented in standard drinking bottles attached to the front of the home cage. Pelleted rat chow was continuously available except during conditioning procedures.

On days 9 and 10, thirty min two-bottle choice tests between CS+ and CS- flavors were conducted in food-deprived or *ad libitum* fed OLETF and LETO rats to assess conditioned preference for the CS solutions in the absence of US(+/-) presentation. Food restricted and *ad libitum* fed choice tests were counterbalanced between groups such that,

on day 8, two experimental groups had chow removed at 1800 hr prior to testing on day 9, while the other two groups were not food-restricted prior to day 9 preference testing. After testing on day 9, plain water and rat chow were returned. Chow was removed at 1800 hr from rats not food-restricted prior to two-bottle choice testing on day 9. On day 10, two-bottle choice testing was performed in rats under the opposite restriction conditions imposed prior to Day 9 choice testing. The left and right side orientation of the two CS flavor solutions was counterbalanced equally between animals.

A parallel experiment was conducted simultaneously with the experiment described above in a separate group of animals (twenty, 10 wk old, naïve 10 wk old rats, weighing 275.4 ± 5.0 g and 228.7 ± 6.2 g, respectively) in which 0.3 M sucrose was replaced with 0.2% non-caloric saccharin solution as the US+ solution. All other protocols were identical. Our final experiment expanded upon the previous two conditioning studies by using both saccharin and sucrose solutions as US solutions. Therefore, in this last experiment, another group of rats (twenty, 10 wk old, naïve 10 wk old rats, weighing 265.9 ± 3.3 g and 222.5 ± 3.7 g, respectively) were conditioned to associate a CS with both a caloric and non-caloric sweet tasting US (0.3 M sucrose: US+; 0.2% Saccharin: US-). All daily access and testing regimens were the same as described in the previous two studies.

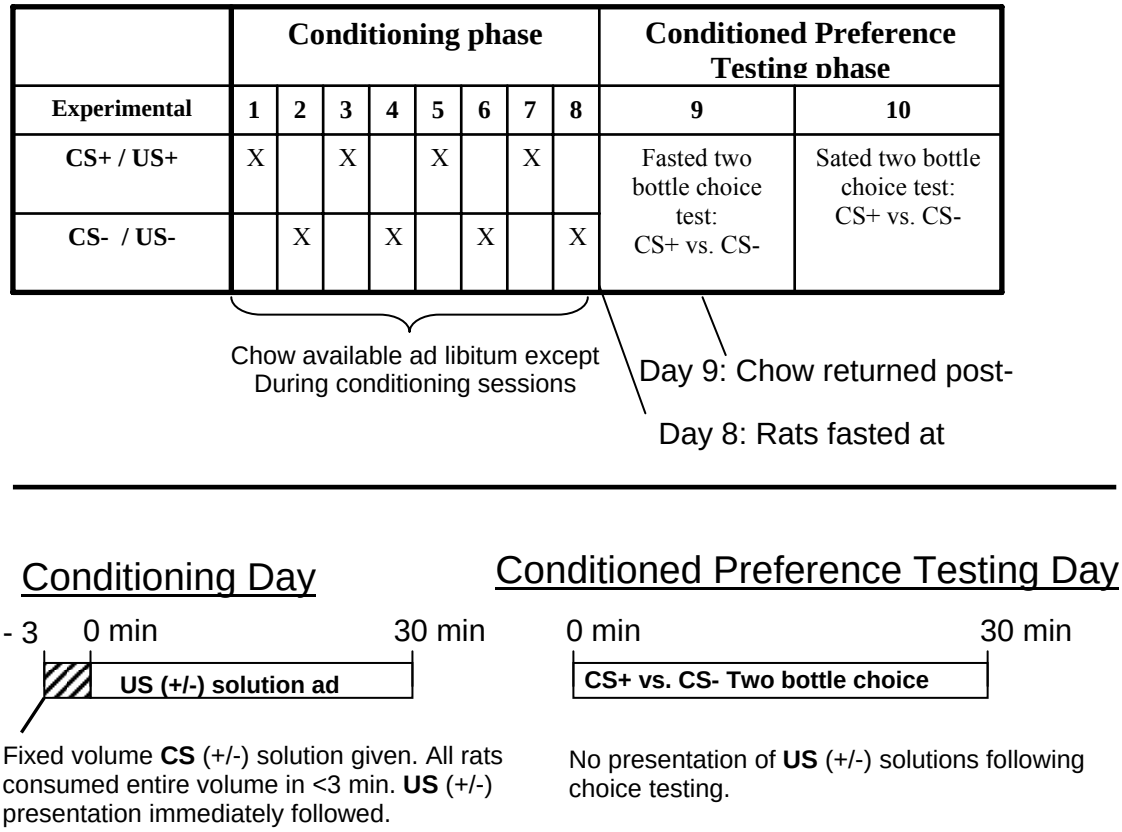


Figure 4.1 Experimental schedule for conditioned preference testing for both study duration (**A**) and within day design (**B**). All three conditioned preference studies conformed to identical procedures, with only US(+/-) solutions altered between studies. Over eight calendar days all rats were exposed to a fixed volume CS solutions immediately preceding 30 min presentation of an assigned US. All animals were given CS+/US+ and CS-/US- pairings on alternate calendar days for a total of 4 exposures to each pairing. Chow was continuously available except during conditioning procedures. On day 8, chow was removed at 1800 hr prior to testing the following day. On day 9, a 30 min two-bottle choice test between CS+ and CS- flavors was conducted to assess conditioned preference for the CS solutions. After testing, water and rat chow was returned for the remainder of the day. On day 10, CS choice testing was repeated with no food restriction.

Statistical Analysis

For sucrose intake studies, one-way repeated measures analysis of variance (rmANOVA) was performed to test within strain effects of food deprivation on subsequent sucrose intake and preference in OLETF or LETO rats. Since OLETF rats displayed increased sucrose intake relative to LETO rats under ad libitum chow feeding conditions, sucrose intake following food deprivation was expressed as a percent increase from sucrose intake when non-deprived on test, which was calculated using the following

formula: Percent Increase = 1-(intake after food restriction (ml)/ intake under ad libitum feeding conditions (ml)) X 100. These results were again analyzed post-hoc by Tukey's honestly significant difference (HSD) test when necessary.

For sucrose preference testing, a two-way rmANOVA was performed with strain and chow deprivation as main effects. Total intakes of both solutions (0.3 M and 1.0 M sucrose) were used to calculate preference percentages according to the following formula: Preference percentage = [volume of 1.0 M sucrose solution (ml) x 100/ total volume of 0.3 M sucrose and 1.0 M sucrose (ml)].

For all conditioned preference studies, US intakes across the conditioning period were analyzed separately according to US solutions using two way rmANOVA with strain and time as main effects. Conditioned preference testing results were analyzed by appropriate one way ANOVA planned comparisons with strain or deprivation state on test as main factor.

All data were expressed as means $\pm$ SEM. Differences were considered statistically significant if $P<0.05$. Statistical analyses were computed with PC-SAS (version 8.02, SAS Institute, Carey, NC).

RESULTS

Intake of 0.3 M and 1.0 M sucrose in OLETF and LETO rats under food-deprived conditions

Results of ANOVA testing for a food deprivation effect on sucrose intake revealed a significant increase in sucrose intake in LETO rats [$F(2,12)=12.94$; $P<0.05$], while OLETF rats showed no such effect ($P=0.124$). Figure 4.2 depicts results of post-hoc analyses showing that after high food restriction, LETO rats significantly increased their 0.3 M sucrose intake relative to non-deprived sucrose intake (16.3 ± 1.1 vs. 20.4 ± 1.4 ml for baseline and low restriction intakes, respectively; $P<0.05$), while both OLETF and LETO rats did not increase intake when under low deprivation conditions (P=ns). Similarly, when 1.0 M sucrose was tested, LETO rats significantly increased their sucrose intake relative to non-deprived sucrose baseline after food deprivation [$F(2,12)=18.42$; $P<0.05$], while OLETF rats again did not (P=ns). Post hoc results depicted in Figure 4.3 show an increase in consumption to have occurred only after high deprivation conditions (9.1 ± 0.9 vs. 12.5 ± 0.4 ml for baseline and low restriction intakes, respectively; $P<0.01$).

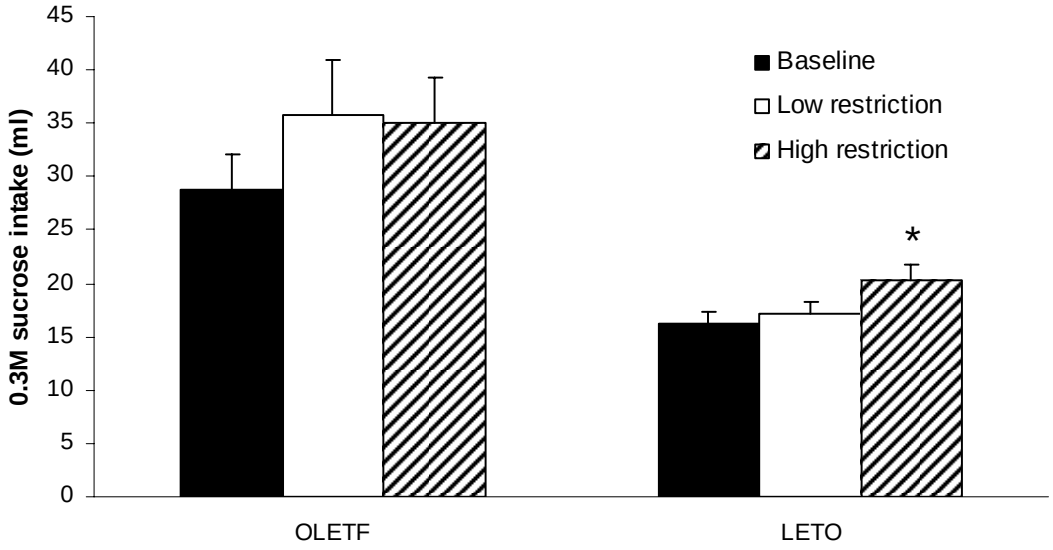


Figure 4.2 0.3 M intake in OLETF and LETO rats after food restriction. When tested after high acute food restriction, LETO rats significantly increased their 0.3 M sucrose intake relative to non-deprived sucrose intake during the same timeframe, while OLETF rats did not. Both OLETF and LETO rats did not increase intake when under low deprivation. (*P<0.01, within strain).

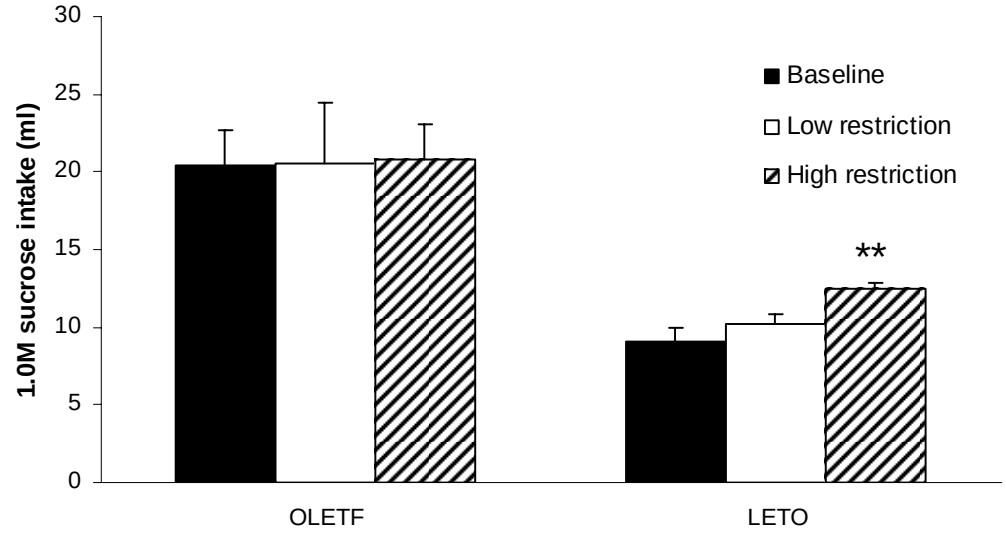


Figure 4.3 1.0 M sucrose intake in OLETF and LETO rats after food restriction. When tested after high acute food restriction, LETO rats significantly increased 1.0 M sucrose (**right panel**) intake relative to non-deprived sucrose intake during the same timeframe, while OLETF rats did not. Both OLETF and LETO rats did not increase intake when under low deprivation. (**P<0.001 within strain).

Preference for 1.0 M vs. 0.3 M sucrose in two-bottle choice test in OLETF and LETO rats under food-deprived conditions

Both OLETF and LETO animals preferred 1.0 M sucrose over 0.3 M sucrose in two-bottle choice testing. One way ANOVA assessing strain effects on sucrose preference showed significantly higher preference for 1.0 M sucrose in OLETF rats when compared to LETO animals [$F(1,12)= 10.24$; $P<0.05$].

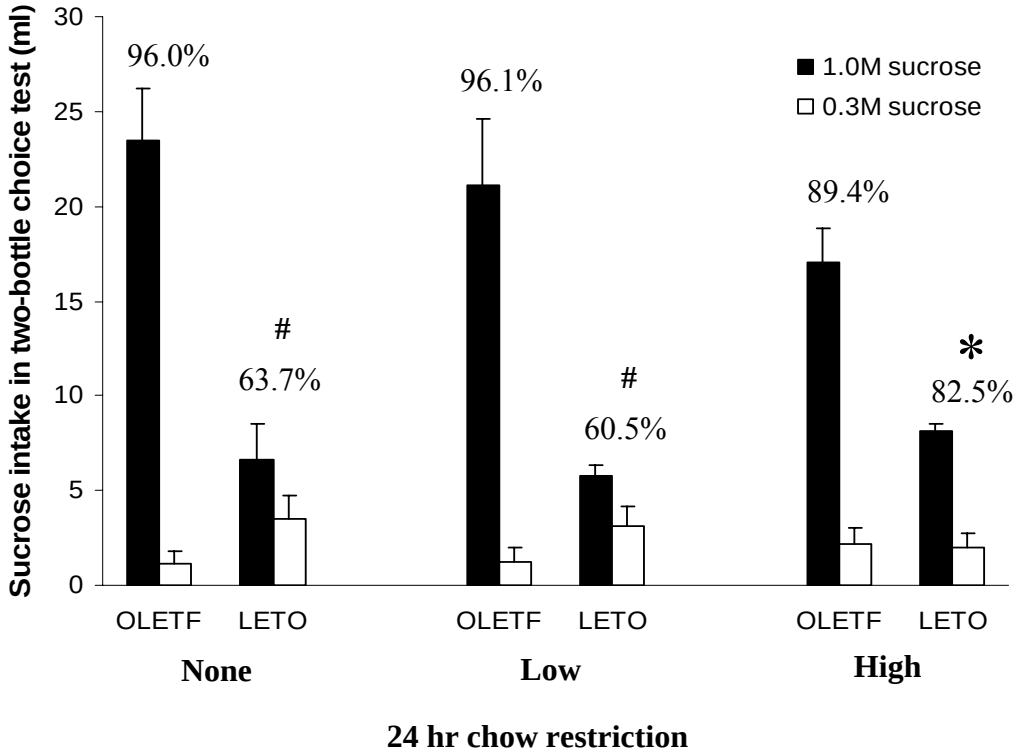


Figure 4.4 1.0 M sucrose preference in OLETF and LETO rats after food restriction. Non-food deprived OLETF rats showed higher preference for 1.0 M sucrose as a percentage of total sucrose intake than LETO rats when given 1 hr access to both 1.0 M and 0.3 M sucrose solutions. LETO rats showed increased 1.0 M sucrose after high food restriction, while OLETF rats showed in difference in sucrose preference under the same restriction conditions. Under low chow restriction, sucrose preference was unaltered in either strain (**$P<0.001$, within strain, $^{\#}P<0.05$ between strains). Numbers above SEM bars represent percent preference between 1.0 M and 0.3 M sucrose for each strain.

As shown in Figure 4.4, OLETF rats showed higher preference for 1.0 M sucrose than LETO rats when non-food deprived (96.1 ± 1.2 % vs. 62.7 ± 12.1 % for OLETF and LETO rats, respectively; $P<0.05$), or under low deprivation conditions (96.0 ± 1.7 % vs.

60.5 ± 12.4 % for OLETF and LETO rats, respectively; $P<0.05$), while no strain difference was noted under the higher restriction condition. Consistent with our preceding results indicating motivational state increases in sucrose intake in LETO rats, LETO rats showed also increased 1.0 M sucrose preference [$F(2,12)= 8.83$; $P<0.05$] when food restricted, while no such response was detected in OLETF rats [Figure 4]. Post-hoc testing showed a significant increase in sucrose preference in LETO rats only after high food restriction ($P<0.05$).

Conditioned preference for sweet solutions in OLETF and LETO rats

There were no overall within group differences in US(+/-) intake during training, or CS(+/-) intake, or preference during testing, according to whether grape or cherry flavor was used as CS+ flavor in all three conditioning studies.. In all experiments, rats consumed all of the 3 ml clamped CS(+/) flavored solutions prior to US(+/-) access. Additionally, no significant differences between results of day 9 or day 10 preference tests were found within deprivation condition across days. Therefore, these data were pooled for subsequent analyses, and presented in the following section accordingly.

Conditioned sucrose vs. water preference in OLETF and LETO rats

Figure 4.5A illustrates intake of US solutions during the 8 day training period. OLETF rats consumed more sucrose (US+) than LETO rats during the 30 min access period [$F(4,18)=33.78$; $P<0.05$]. Post-hoc analyses showed this effect across all four days of presentation (largest $P<0.05$). Water (US-) intake was not significantly different between strains. Figure 4.5B shows results of conditioned preference testing after all rats

were given a 30 min two-bottle choice test between CS+ and CS- flavored solutions under either food-deprived or non-deprived conditions. OLETF rats preferred the CS+ flavor to a greater extent than LETO rats under both deprived [82.9 ± 4.9 % and 70.8 ± 5.4 % for OLETF and LETO rats, respectively: $F(1,18)= 13.08.$; $P<0.05$] and non-deprived conditions [80.9 ± 5.3 % and 53.6 ± 7.5% for OLETF and LETO rats, respectively: $F(1,18)= 39.81$; $P<0.01$]. When examining the effect of motivational state on conditioned sucrose preference, LETO rats exhibited a significantly increased CS+ preference when food deprived on test [$F(1,18)=19.08$; $P<0.01$], in contrast to OLETF rats which did not show altered preference for CS+ after overnight food deprivation ($P=0.726$).

Conditioned saccharin vs. water preference in OLETF and LETO rats

Figure 4.6A shows intake of US solutions across the 8-day training period. OLETF rats consumed more of the non-caloric sweet saccharin (US+) than LETO rats during the 30-min access period directly following CS+ consumption [$F(4,18)=26.78$; $P<0.05$]. Post-hoc analyses revealed intake to be persistently higher in OLETF rats compared to LETO rats each day of presentation (largest $P<0.05$). Water (US-) intake was again not significantly different between strains. Results of conditioned saccharin preference testing are presented in Figure 4.6B. Under conditions identical to those imposed in the previous experiment, OLETF rats preferred the CS+ flavor to a greater extent than LETO rats under only non-deprived conditions [76.4 ± 4.3 % and 57.5 ± 5.9 % for OLETF and LETO rats, respectively: $F(1,18)= 28.66$; $P<0.01$], while no significant difference was noted between strains under deprived conditions (P=ns).

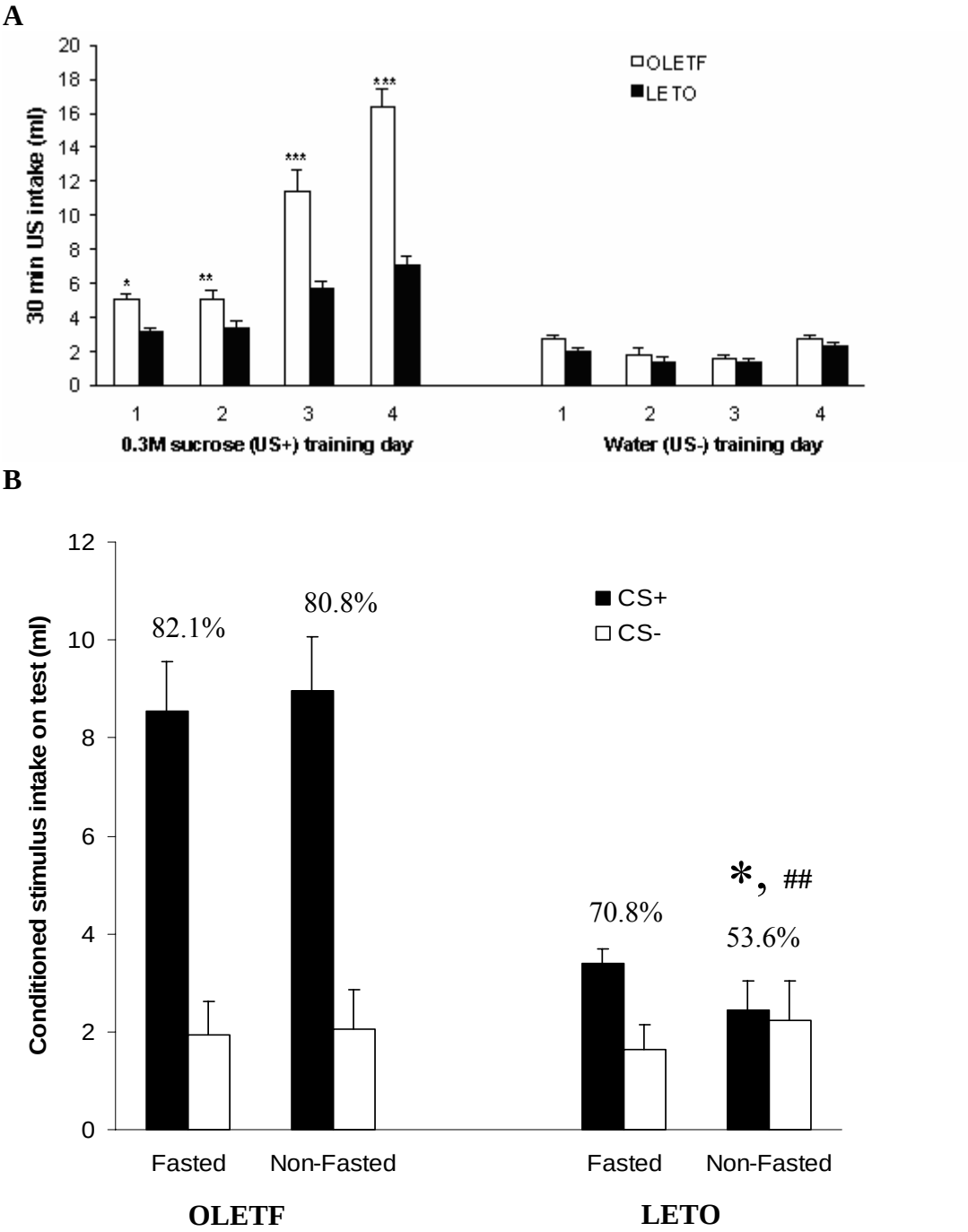


Figure 4.5 Raw 0.3 M sucrose (US+) and water (US-) intakes during one bottle conditioning phase (**A**) and percent preference for CS+ during two-bottle CS+ vs. CS- choice tests in food-deprived or non-deprived OLETF and LETO rats (**B)**. After consuming a fixed 3 ml volume of flavored saccharin (CS), OLETF rats consumed more sucrose (US+) than LETO rats during a 30 min access period across all four days of presentation. Water (US-) intake was not significantly different between strains (**A**). When given a 30 min two-bottle choice test between both CS+ and CS- solutions, OLETF rats preferred the n CS+ flavor to a greater extent than LETO rats regardless of motivational state on test. In contrast, OLETF rats did not show altered preference for CS+ after overnight food deprivation, whereas LETO rats exhibited a significantly increased CS+ preference when food deprived on test (**B**) (*P<0.05,**P<0.01,***P<0.001 within strain: $^{\#}$P<0.05, $^{\#\#}$P<0.01 between strains). CS+ preference percentages are illustrated above SEM bars for raw CS (+/-) intakes within each group.

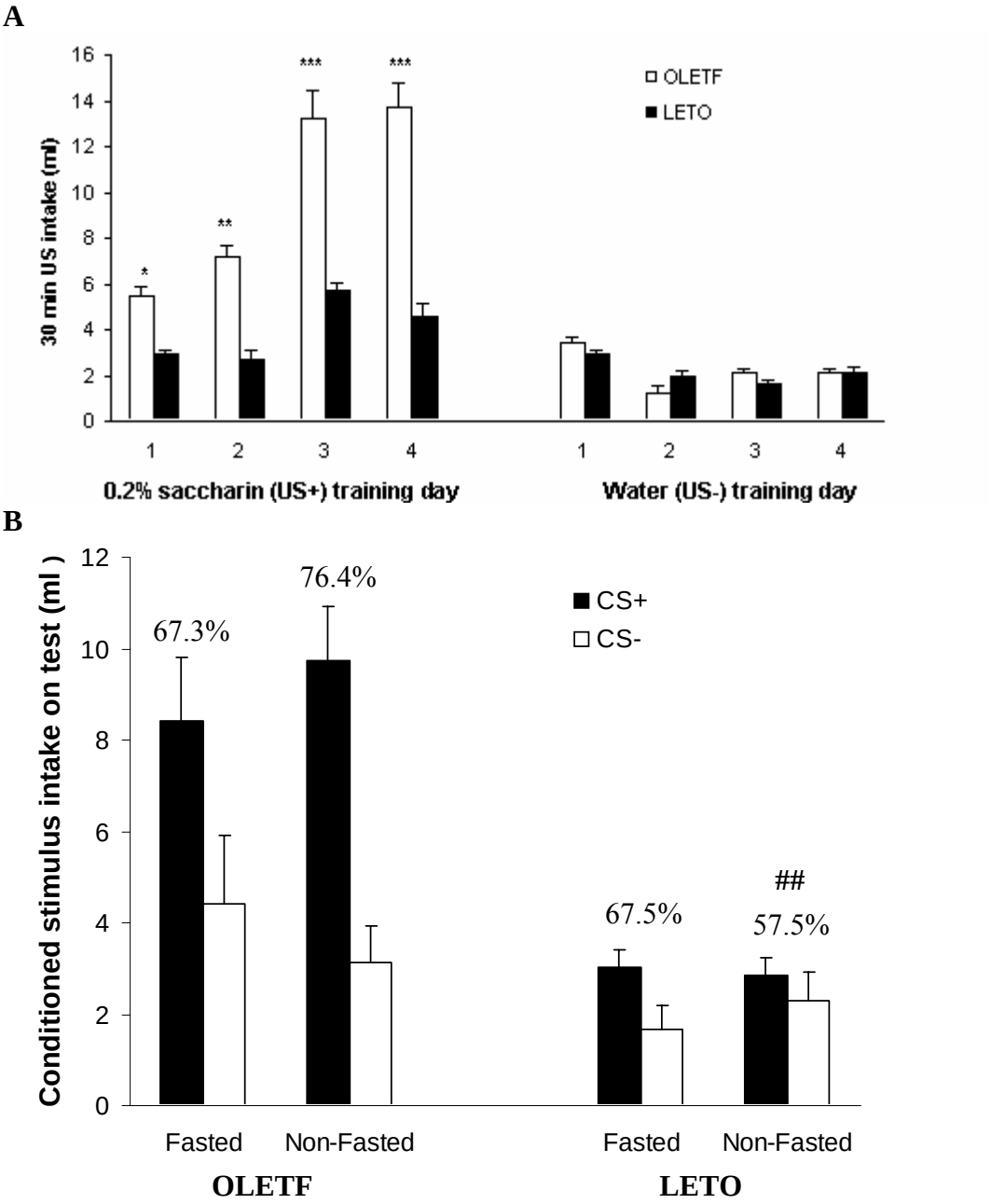
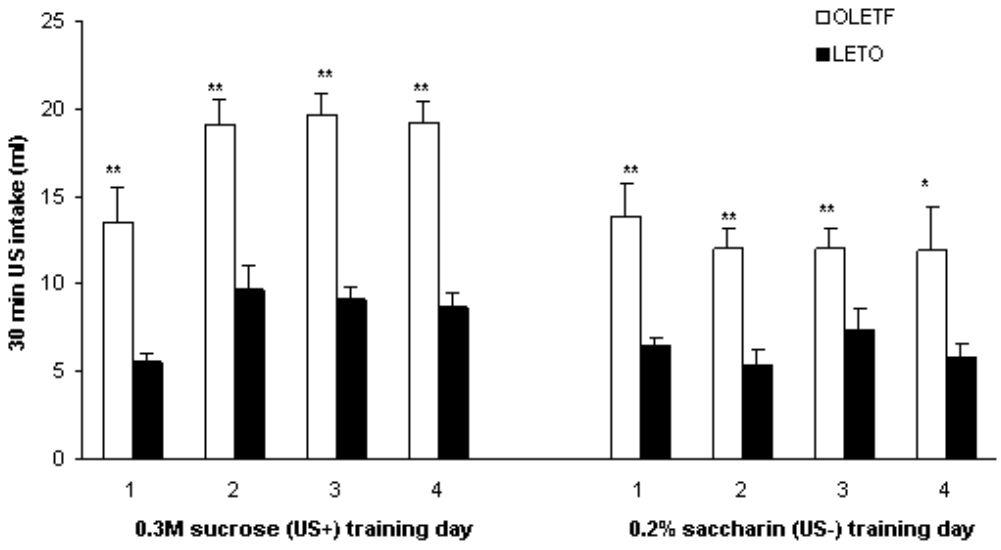


Figure 4.6 Intakes of 0.2% saccharin (US+) and water (US-) during one bottle conditioning phase (**A**) and preference percentages for CS+ during two-bottle CS+ vs. CS- choice tests in non-deprived or food-deprived OLETF and LETO rats (**B**). After consuming a fixed 3 ml volume of flavored saccharin (CS), OLETF rats consumed more saccharin (US+) than LETO rats during a 30 min access period across all four days of presentation. Water (US-) intake was not significantly different between strains except on the first day of training (**A**). When given a 30 min two-bottle choice test between both CS+ and CS- solutions, OLETF rats preferred the CS+ flavor to a greater extent than LETO rats when food deprived on test. No strain differences were noted in CS+ preference in the absence of food restriction on test. Both LETO and OLETF rats did not show altered preference for CS+ within strain after overnight food deprivation (**B**) (*P<0.05,**P<0.01,***P<0.001 within strain: $^{\#\#}$P<0.01 between strains). CS+ preference percentages are given above SEM bars for raw CS (+/-) intakes within each experimental group.

In addition, LETO rats also exhibited no significant increase in CS+ preference when food deprived on test (P=ns) relative to non-deprived preference. Similarly, OLETF rats showed comparable preference for CS+ regardless of motivational state on test (P=ns).

Conditioned sucrose vs. saccharin preference in OLETF and LETO rats

Results of raw intakes during sucrose (US+) and saccharin (US-) conditioning periods are shown in Figure 4.7A. ANOVA results showed that OLETF rats consumed significantly greater amounts of both sucrose (US+) [$F(4,18)=24.96$; $P<0.05$] and saccharin (US-) [$F(4,18)=17.45$; $P<0.05$] solutions during training than LETO rats during the 30 min access period directly following CS(+/-) consumption. Post-hoc analyses revealed intake to be persistently higher in OLETF rats compared to LETO rats each day of presentation for both US+ and US- solutions (largest $P<0.05$ for both US+ and US-). Conditioned preference testing revealed the sucrose (US+) associated flavor (CS) to be the most preferred. Thus, the results of conditioned preference testing are presented in terms of CS+ preference. Figure 4.7B depicts results showing OLETF rats preferred the CS+ flavor to a greater extent than LETO rats under only non-deprived conditions [87.6 ± 4.4 % and 57.9 ± 4.5 % for OLETF and LETO rats, respectively: $F(1,18)= 31.19$; $P<0.01$], while no significant difference was noted between strains under deprived conditions (P=ns). In addition, LETO rats exhibited a significantly increased CS+ preference when food deprived on test [$F(1,18)=25.39$; $P<0.01$] relative to non deprived preference, whereas OLETF rats showed similar preference for CS+ regardless of motivational state on test (P=ns).



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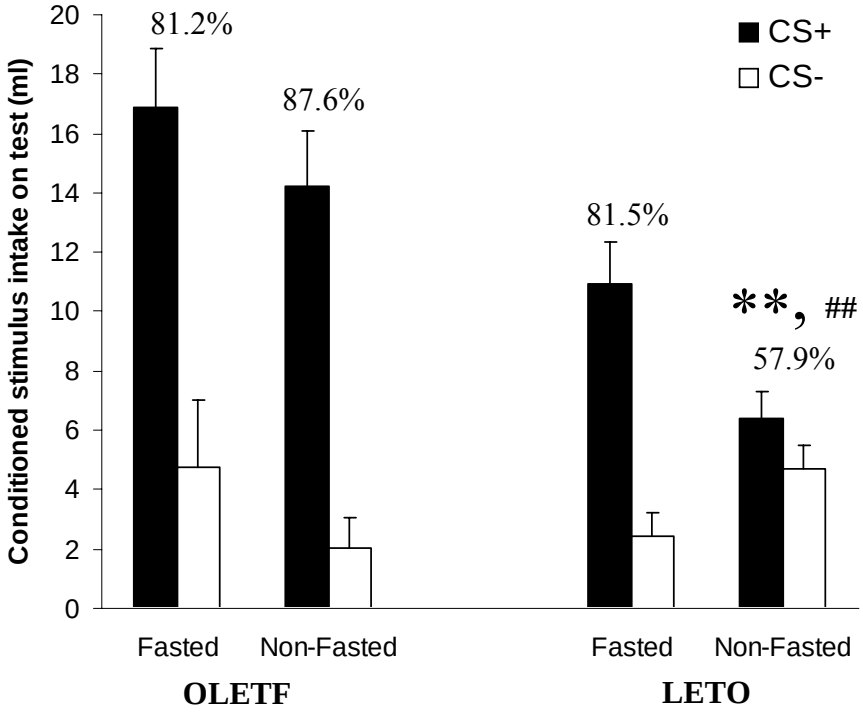


Figure 4.7 Intakes of 0.3 M sucrose (US+) and 0.2 % saccharin (US-) solutions during one bottle conditioning phase (**A**) and preference percentages for CS+ during two-bottle CS+ vs. CS- choice tests in non-deprived or food-deprived OLETF and LETO rats (**B**). OLETF rats consumed more sucrose (US+) and saccharin (US-) than LETO rats during a 30 min access period across all four days of presentation after consuming a fixed volume of a flavored saccharin (CS), (**A**). When given a 30 min two-bottle choice test between both CS+ and CS- solutions, OLETF rats preferred the CS+ flavor to a greater extent than LETO rats when food deprived on test. No strain differences were noted in CS+ preference in the absence of food restriction on test. In contrast, OLETF rats did not show altered preference for CS+ within strain after overnight food deprivation, whereas LETO rats exhibited a significantly increased CS+ preference when food deprived on test (**B**) (*P<0.05,**P<0.01,***P<0.001 within strain: $^{\#\#}$P<0.01 between strains). CS+ preference percentages are given above SEM bars for raw CS (+/-) intakes within each experimental group.

DISCUSSION

The present results show that OLETF rats exhibit clear deficiencies in modifying sucrose intake after periods of acute food deprivation compared with LETO controls. For both a relatively dilute and concentrated sucrose concentration, OLETF rats show no significant alterations in either sucrose intake or preference, while LETO rats show both increased sucrose intake and preference under food deprivation conditions. Similarly, OLETF rats exhibit a higher conditioned flavor preference for sucrose relative to LETO, regardless of deprivation state. When OLETF and LETO rats are conditioned to prefer a saccharin solution, OLETF rats show a higher preference for the saccharin associated flavor relative to LETO rats when non-deprived, however neither strain shows differential flavor preference for saccharin according to deprivation state on test. These results suggest that orosensory effects of palatable solutions impart a greater role in governing food associations in OLETF rats relative to control, non-mutant LETO rats.

The size of a meal is partly dictated by the manifestation of post-ingestive inhibitory feedback signals. Disruptions of these signals can lead to overconsumption and weight gain. Indeed, we as well as others have shown that OLETF rats suppress intake less than LETO controls in response to a variety of intestinal nutrient infusions (Covasa and Ritter 2001), as well as gastric fat preloads (Schwartz et al. 1999). Also, OLETF rats reduce sucrose sham feeding less than LETO rats following gastric balloon distention (De Jonghe et al. 2006) suggesting that mechano-detection may be also impaired in these animals. While there is no doubt that aberrations in these signaling mechanisms play a major role in leading to excessive meal size (Moran et al. 1998), the amount consumed within a meal can also be modulated by the feed-forward effects resultant from

orosensory stimulation (Smith 1996). In this regard, we have recently reported ingestive abnormalities in the OLETF rats characteristic of both enhanced taste sensitivity and orosensory controls of sucrose intake which are manifest in the absence of gastric/post-gastric satiation effects (De Jonghe et al. 2005; Hajnal et al. 2005). In fact, non food-deprived, pre-diabetic, OLETF rats will sham feed more of a concentrated sucrose solution than age-matched LETO rats, thus overconsuming sucrose in the absence of post-absorptive effects of sucrose (De Jonghe et al. 2005). Furthermore, recent data from our laboratory have shown OLETF rats to exhibit heightened lick responsiveness to various sweet stimuli in short access tests indicative of a generalized increase in sweet taste preference (Hajnal et al. 2005). The possibility that central taste functions are impaired in the OLETF rat, including an increased sensitivity to the incentive motivational effect (i.e. palatability) of ingestive stimuli is further supported by the results reported here. The observation that the magnitude of sucrose intake is similar in both non-deprived and 24 hr food-deprived OLETF rats suggests that an increase in orosensory sensitivity that is already present in ad libitum-fed conditions could be a major contributor to enhanced sucrose intake in addition to the presence of satiation deficits. Specifically, free feeding OLETF rats are known to overconsume rat chow by 30% relative to age-matched LETO rats (Moran et al. 1998). However, when given access to a palatable sucrose solution, the degree of hyperphagia observed in non food deprived OLETF rats increases by almost 60% for 0.3M sucrose and 146% for 1.0M sucrose relative to freely fed LETO controls. Barring differences between liquid and solid foods on this effect, the augmented magnitude of sucrose hyperphagia relative to chow consumption suggests that other than caloric properties of the sucrose, i.e. palatability,

exacerbate the hyperphagic behavior of the OLETF rat. It could be argued that the failure of OLETF rats to increase sucrose intake when deprived may have been due to a ceiling effect if they drank large amounts when non-deprived. Similarly, the failure of the OLETF rats to increase 1.0M sucrose preference might be due to their high preference when non-deprived. However, in our previous work OLETF rats were able to drink more than 26 ml of 12.5% glucose solution within 30 min (Covasa and Ritter 2001) . Therefore, we can safely assume that in our study during the 60-min recording period, OLETF would have been able to increase their intake beyond the amount consumed under deprivation. Taken together, these emerging results suggest that gustatory sensitivities to sweet tastants may also significantly contribute to known satiation deficits leading to the hyperphagic phenotype of the OLETF rat.

It is well known that the post-ingestive, or nutritive properties of nutrients are able to condition flavor preference (Sclafani 2004; Sclafani and Ackroff 2004). By assessing flavor preference in food-deprived or satiated animals previously conditioned to associate the flavor of a non-caloric CS with presentation of a palatable US, we may ascertain the relative extent to which a CS preference associated with sucrose is due to an association with the sweet taste of sucrose, or it's post-ingestive/nutritive value. Previous work has focused on the effects of food deprivation on conditioned flavor preference in normal (i.e. non-mutant) rats using similar degrees of food deprivation employed in the current design (Capaldi and Myers 1982; Capaldi et al. 1983; Capaldi et al. 1987). However, several of these studies have examined specifically how flavor preferences are modulated by food deprivation using food exposures during conditioning phases that have been previously associated with a specific caloric need state before preference testing, i.e. low or high

food deprivation conditions. In all three of our flavor conditioning tests, CS solutions and the associated tastants (US) were presented when the animal was under *ad libitum* fed conditions prior to testing. Thus, our design aimed at the effects of differential caloric motivational state specifically on the expression of conditioned flavor preferences and not the acquisition of these preferences (i.e., ad libitum chow availability throughout the conditioning phase).

In our first flavor conditioning test, we showed that food-deprived OLETF rats exhibit no difference in conditioned flavor preference for sucrose when either ad libitum fed or food-deprived on test, while LETO rats showed significantly increased flavor preference for sucrose when food-deprived on test. These results support our hypothesis that while in LETO rats a calorie-association controls preference for sucrose over taste-association under deprived conditions, in OLETF rats the performance on test is unresponsive to hunger level. We also showed that when comparing between strains, OLETF rats exhibit a higher flavor preference for sucrose relative to LETO rats regardless of deprivation state. Such results may be indicative of a generalized stronger degree of sucrose preference formation in OLETF relative to LETO rats. One possible explanation for this effect is that, among OLETF rats, sucrose possesses an inherently greater reinforcing effect as unconditioned stimulus (Reilly 1999; Sclafani and Ackroff 2003). Such an effect may be related to differences in gustatory functions or the sensitivity of the reward systems, both of which aspects are currently under investigation in our laboratory. While beyond the scope of the present design, investigation of the reinforcing effects of sucrose through operant conditioning procedures may support this notion more directly. Nonetheless, we are still able to provide clear evidence that when

tested for conditioned preference, increased caloric need due to food deprivation appears to play a minimal role in the expression of conditioned sucrose preference in the OLETF rat. In other words, orosensory effects of sucrose in hyperphagic OLETF rats are stronger to form preferences than in LETO rats, irrespective of deprivation state.

Our results of enhanced conditioned saccharin preference in non-deprived OLETF rats compared to LETO controls further support this hypothesis. As saccharin is a non-caloric sweet tastant, nutrient satiation deficiencies in the OLETF rat could not account for increased saccharin intake or preference in these animals. We have recently published evidence of enhanced lick rate of saccharin solutions in the OLETF rat in brief 10 s access testing (Hajnal et al. 2005). Thus, one explanation for increased saccharin intake during our conditioning phase is an enhanced gustatory sensitivity for the sweet taste of saccharin. Finally, neither strain showed significant alterations in conditioned preference for saccharin according to deprivation state on test consistent with previous reports of conditioned saccharin preference (Fedorchak and Bolles 1987). This finding lends support to the notion that an increased potential of sucrose to form conditioned preferences is based on its caloric predictive value or metabolic effects. This possibility can be addressed by examining sham feeding of sucrose during conditioning.

Our final study showed that when replacing water (US-) with a more palatable non-caloric sweet saccharin solution, we obtained similar results of conditioned sucrose -(US+) preference as that of our earlier experiment. Specifically, OLETF rats showed a higher conditioned preference under non-deprived conditions relative to LETO rats. Secondly, OLETF rats were unresponsive to changes in motivational state on test, while LETO rats showed a significant increase in conditioned sucrose preference response to

food deprivation on test. This study suggests that while control LETO rats increase their conditioned preference for a caloric sweet taste over one that is non-caloric when food–deprived, OLETF rats appear insensitive to these effects.

Across conditioning experiments, we observed increased US intakes in OLETF rats relative to LETO rats. It is possible that a larger US consumption during training by OLETF rats may result in a non-equal US-CS transfer strength that, in turn, may lead to differential CS intake on test when assessing expression of conditioned flavor preference. Such an assumption, however, can not be conclusively made. For example, there are data suggesting that increased US exposure, or consumption, during training does not necessarily correlate with conditioned preference in two-bottle choice tests (Lucas et al. 1997). Furthermore, OLETF rats over-consume sucrose under real-feeding conditions in part due to reduced satiation signaling, suggesting that increased US intake under *ad libitum* access conditions is not due primarily to increased US exposure effects, but rather is an artifact of reduced negative feedback signals normally limiting intake within this setting. If our results of increased conditioned preference for sweet solutions in OLETF rats were due to higher intakes during training, we would expect an overall generalized increase in conditioned sweet preference in OLETF rats across all test conditions. However, OLETF and LETO rats did not differ in CS+ preference when food-deprived in all three of our conditioned preference testing experiments, suggesting that heightened preference in the OLETF is not simply due to higher intakes during training.

It may also be possible that the presented findings are due to deficiencies in the integration of post-ingestive and orosensory signals determining meal size. Recent work from our laboratory has demonstrated that CCK-1 receptor-containing neurons mediating

vagally transmitted peripheral CCK signaling in the nucleus of the solitary tract, area postrema, and dorsal vagal motor nucleus are virtually absent in the OLETF rat (Covasa and Ritter 2005). These data confirm that CCK-related satiation deficits in the OLETF extend to regions of the hindbrain known to relay satiation signals form the gut to the brain. A separate body of work has also shown that the absence of CCK-1 receptors in the dorsomedial hypothalamus may be responsible for altered NPY expression in the obese OLETF rat (Bi et al. 2001; Moran et al. 2002). Of particular interest to the current results are our recent reports that OLETF rats exhibit enhanced intake of sucrose when post-ingestive effects of the solution are eliminated or largely minimized (De Jonghe et al. 2005; Hajnal et al. 2005).

It is also possible that deficits in food reward processing could help explain enhanced orosensory control of sweet preference. Emerging data suggest that dopamine activity, particularly within the mesolimbic pathway, increases after ingestion of palatable sucrose solutions (Wise 2002; Hajnal et al. 2004). In similar paradigms using non-mutant animals, a clear positive relationship has been shown between dopamine release in the nucleus accumbens and ingestion of palatable sucrose solutions in designs when orosensory components of these tastants are isolated (Wise 2002; Hajnal et al. 2004). Furthermore, we have shown an altered D2 dopamine receptor function related to sucrose intake in addition to lower D2 receptor binding in the striatum of pre-diabetic OLETF rats of similar age (Margas 2005). Additionally, our laboratory and others have also reported evidence of alterations in higher dopamine functions such as the inhibition of acoustic startle response and sensorimotor gating which further indicate possible dopamine associated deficits in the OLETF rat (Feifel et al. 2001; De Jonghe et al. 2005).

These neural alterations could function to enhance the effects of the hyperphagia in the OLETF rat through not only independent mechanisms, but also collectively, resulting in an overall augmentation of meal size.

Taken together, the present studies indicate that among OLETF rats, both intake of and preference for sucrose, as well as a flavor conditioned sucrose preference, has a relatively stronger orosensory component compared to control LETO animals. Furthermore, food deprivation conditions, which lead to increased consumption of sucrose or sucrose associated flavors in LETO rats, appear to have little impact on

CHAPTER 5

BRIEF INTERMITTENT ACCESS TO SUCROSE DIFFERENTIALLY MODULATES PREPULSE INHIBITION AND ACOUSTIC STARTLE RESPONSE IN OBESE CCK-1 RECEPTOR DEFICIENT RATS

INTRODUCTION

Cholecystokinin (CCK) is one of the most widely studied endogenous gut peptides controlling satiation signaling and feeding behavior in rodents (see (Ritter 2004) for review). It is well established that of the two known CCK receptors, CCK-1 and CCK-2, the inhibitory feeding effects of CCK are mediated mainly via CCK-1 receptors (Moran et al. 1992). In addition to its role in peripheral satiation signaling, CCK found within specific brain regions can play a modulatory role in the activity of several neurotransmitters, including dopamine (DA). Specifically, CCK stimulates DA release by acting on CCK-1 receptors (Crawley 1991), while activation of the CCK-2 receptor confers an inhibitory effect on DA release (Marshall et al. 1991). In contrast to the ubiquitous presence of CCK-2 receptors throughout the brain, populations of central CCK-1 receptors are only known to exist within limited regions, such as the dorsomedial hypothalamus (Moran et al. 1986; Woodruff et al. 1991), striatum (Graham et al. 1991), with specifically high intensity in the caudo-medial shell of the nucleus accumbens (NAcc) (Heidbreder et al. 1992; Lanca et al. 1998). In the caudal NAcc, DA and CCK are co-released in vivo after administration of drugs that increase DA neuronal firing rate (Crawley 1991; Ladurelle et al. 1994). A similar co-release of DA and CCK in the

somatodendritic area of the mesoaccumbens DA system, in the ventral tegmental area (VTA) has been shown to affect DA cell firing rate (Hamilton and Freeman 1995).

Natural and transgenic CCK receptor mutant animal models offer a unique opportunity for studying interactions between DA and CCK systems. The Otsuka Long-Evans Tokushima Fatty (OLETF) rat is an outbred strain of Long-Evans Tokushima (LETO) lacking expression of the CCK-1 receptor due to a recently discovered spontaneous 6.8 kb deletion in the CCK-1 receptor gene (Takiguchi et al. 1997). Therefore, the OLETF rat represents a potentially important model to assess mesolimbic DA transmission *in vivo* where effects of CCK-1 receptor activation are absent. Indeed, recent data from Feifel and colleagues have demonstrated that OLETF rats exhibit enhanced amphetamine (AMPH) and cocaine -induced increases in NAcc DA release relative to LETO controls (Feifel et al. 2003).

Prepulse inhibition (PPI) of acoustic startle reflex (ASR), where a brief loud burst of noise is immediately preceded (50-500 msec) by softer non-startling sound, has been previously recognized as an operational measure of sensorimotor gating (Swerdlow et al. 2001). The mesolimbic DA pathway is one of the crucial systems in the brain that contributes to PPI response (Swerdlow et al. 1992). Specifically, DA agonists such as AMPH can disrupt PPI by inhibiting the normal reduction of startle response due to the prepulse (Mansbach et al. 1988). Participation of CCK-1 receptors in mediating PPI responses has also been documented. For example, cerulein, a CCK agonist with preferentially affinity for CCK-1 receptors partially reverses AMPH-induced reductions in PPI (Feifel et al. 1999). The CCK-1 receptor antagonist, devazepide, but not the CCK-2 antagonist, L-365,260, blocked cerulein's effect on PPI, suggesting CCK-1 receptor

mediation of CCK effects on PPI (Feifel et al. 1999). Furthermore, CCK-1 receptor deficient OLETF rats are unresponsive to AMPH-induced decreases in PPI compared to LETO rats, which show a disruption of PPI consistent with AMPH treatment in non-mutant animals (Feifel et al. 2001).

Our laboratory has recently demonstrated that OLETF rats have an increased preference for both real and sham feeding of sucrose solutions (De Jonghe et al. 2005). The effects of sucrose on dopaminergic transmission are well recognized. Sham feeding sucrose in rats triggers NAcc DA release as a function of sucrose concentration (Hajnal et al. 2004). When given intermittent access to sugar solutions, non-mutant rats exhibit increased binding of D1/D5 DA receptors in the NAcc (Colantuoni et al. 2001). Despite the emerging indication of enhanced DA signaling through sucrose consumption, only the effects of sucrose on ASR have been addressed (Desousa et al. 1998), where high sucrose feeders exhibit altered ASR relative to low sucrose feeders in response to acoustic stimuli. The impact of sucrose treatment on PPI remains unknown. Furthermore, the role of CCK-1 receptors in mediating ASR and PPI responses following repeated sucrose consumption has not been examined.

Therefore, the goal of the present study was twofold: 1) to test the effects of repeated brief sucrose access on ASR and PPI responses in general; and 2) to determine the specific involvement of CCK-1 receptor in this modulation by assessing strain differences in startle responses in OLETF and LETO rats after sucrose consumption.

METHODS

Subjects:

Fourteen-week old male OLETF and LETO rats were obtained as a generous gift of the Tokushima Research Institute, Otsuka Pharmaceutical, Tokushima, Japan. All animals were individually housed in mesh-floored, stainless-steel hanging cages in a temperature-controlled vivarium while maintained on a constant 12:12-h light-dark cycle (lights on at 0600). Rats were handled daily for a minimum of one week prior to the onset of experimental procedures. Tap water and pelleted rat chow (Purina 5001) were available ad libitum throughout experiments. All protocols used were approved by The Pennsylvania State University Institutional Animal Care and Use Committee.

Procedures and experiments

Habituation to Startle Chamber

Twenty rats (10 OLETF and 10 LETO) with average body weights of 452.6 ± 10.84 g and 367.5 ± 2.3 g, respectively, were divided in two groups (n=5 per strain) matched for body weight within each strain. All rats were acclimated to an SR-LAB (San Diego Instruments, San Diego, CA) acoustic startle chamber (ASC) three times prior to experimental onset. Rats within the same stimulus group were run on the same day, i.e. sucrose (SUC) rats (n=5) alternated daily with control, non-sucrose (NON), rats (n=5).

Sucrose Training

For sucrose regimen, we have adapted a protocol from our previous studies that demonstrated potency of brief, daily 20-min sucrose access on altering feeding behavior as well as cause functional and histological alterations in dopamine markers as early as after 6 days (Hajnal and Norgren 2002). Following habituation to the startle chamber, SUC rats within each strain were trained to drink 10.0 ml of 0.3 M (10.3% w/v) sucrose during a brief 20 min access period, while the NON group never received sucrose access at any time. Three additional days of sucrose training occurred once each SUC rat consumed all 10 ml within the 20 min allotted period. Sucrose was presented at either 0900, 1000, or 1100 hr during these three training days in order to dissociate any specific time cues of expectation associated with sucrose access. Starting at 0800 hr on training day 4, all rats were tested in startle chambers. For SUC rats, this occurred immediately following sucrose access, while NON rats were tested at simultaneous intervals without sucrose access. Each rat was presented with 10 ml of 0.3 M sucrose. Immediately following consumption, the rat was placed in the ASC for testing. Rats within treatment groups and strain were randomly selected for time of testing.

Acoustic Startle Protocol

For testing ASRs, the rats were placed in the startle chamber for a 5 min acclimation period with a 65 dB background noise. Each rat was tested according to the same pre-determined pseudo-randomized sequence of startle trials. The trial series consisted of: 1) a 120 dB, 40 msec noise burst presented alone, or 2) the same 120 dB, 40 msec noise burst preceded 100 msec by one of three prepulses (20 msec noise burst) that were 4,8, or 12 dB above background. Each test session consisted of 7 presentations of

the 4 experimental trials as described above, presented in a pseudo-randomized order. Variable inter-trial intervals averaged 8 sec (range 4-12 sec). The mean value of all 7 trials within each pulse-pairing was used as the rat's value for each pulse-pairing. If the number of usable trials within a pulse-pairing was less than 4, then that pulse-pair was excluded from subsequent analyses.

Statistical Analysis

The startle response computer output file contains two main values of interest: Vmax and Tmax. Vmax is the maximum amplitude spike that occurs during each trial, while Tmax is the time at which this value is recorded. In our protocol, Tmax occurs at ~20 msec (for pulse alone) or ~120 msec (for all prepulse-pulse pairings) after trial recording begins, which corresponds to the timeframe immediately following the 120 dB acoustic stimuli at 20 msec after recording (pulse alone trial) or the 120 msec mark after recording (for all pre-pulse paired trials) . In a few cases, Tmax values occurred prior to the initiation of any acoustic stimuli, indicating that the displacement measured was not due to the acoustic stimuli , and thus was not a measure of startle response. In all, less than 10 such cases were recorded for individual trials, appearing randomly among the four experimental groups. These data were excluded from subsequent analyses.

PPI is defined as the percent reduction in startle amplitude in the presence of the prepulse stimulus compared to the amplitude in the absence of the prepulse stimulus [100-(100 x amplitude on prepulse trial/amplitude on 120 dB pulse trial alone)]. ASR and PPI were calculated by two-way repeated measures analysis of variance (rmANOVA) with strain and sucrose treatment as main factors. ANOVA results were subsequently analyzed by Tukey's honestly significant difference (HSD) test post-hoc tests when

applicable. All data are expressed as means ± SEM. Differences were considered statistically significant if $P<0.05$. All statistical analyses were carried out with PC-SAS (version 8.02, SAS Institute, Carey, NC).

RESULTS

A significant interaction [$F(3,16)=4.84$; $P<0.05$] of strain x sucrose access on acoustic startle response was observed. As shown in Figure 5.1, there were no significant difference in baseline (i.e., with no sucrose) ASR between OLETF and LETO rats (137.5 ± 26.1 mV and 224.3 ± 37.0 mV for LETO and OLETF rats, respectively), although there was a trend toward higher ASR in Lefts (P=ns). In contrast, in animals receiving brief access to sucrose, a significant genotype main effect [$F(1,16)=6.84$; $P<0.01$)] was observed as OLETF rats receiving sucrose had significantly higher ($P<0.01$) ASR than LETO rats receiving sucrose (174.6 ± 21.8 mV and 364.7 ± 53.0 mV for LETO and OLETF rats, respectively).

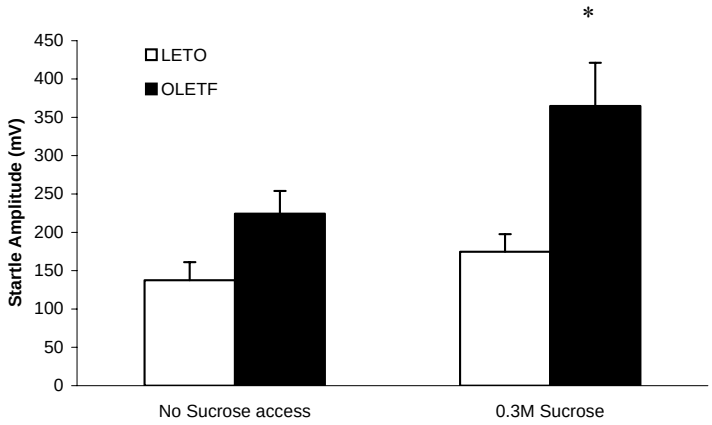


Figure 5.1 OLETF rats receiving sucrose had a higher ASR magnitude than LETO rats receiving sucrose. In contrast, there were no differences in baseline ASR between OLETF and LETO rats. (*P<0.05 between strains).

This experiment also yielded a significant genotype x sucrose treatment interaction on prepulse inhibition [F(3,16)=7.93; P<0.02]. Figure5.2A shows that while non-sucrose receiving controls (NON) exhibited statistically identical prepulse inhibition percentages (45.5 ± 5.8 % and 47.7 ± 2.3 % for LETO and OLETF rats, respectively). LETO rats receiving sucrose treatment had a significantly lower PPI percentage (P<0.01) than OLETF rats receiving the same treatment (32.4 ± 2.3 % and 57.7 ± 3.1 % for LETO and OLETF rats, respectively). Additionally, sucrose receiving LETO rats had a significantly lower (P<0.05) PPI percentage than non-sucrose controls (32.4 ± 2.3 % and 45.5 ± 5.8 % for sucrose and non-sucrose treatments, respectively). No significant sucrose effects on overall PPI (P=ns) were noted in OLETF rats (57.7 ± 3.1 % and 47.7 ± 2.3% for sucrose and non-sucrose treatments, respectively). Figure 5.2B illustrates the results of post-hoc analyses which revealed that LETO rats exhibited significantly reduced PPI across all three levels of prepulse intensity [P<0.05, for PPI-4 and PPI-8 trials; P<0.01, for PPI-12 trials], while OLETF rats showed no response [all P's =ns].

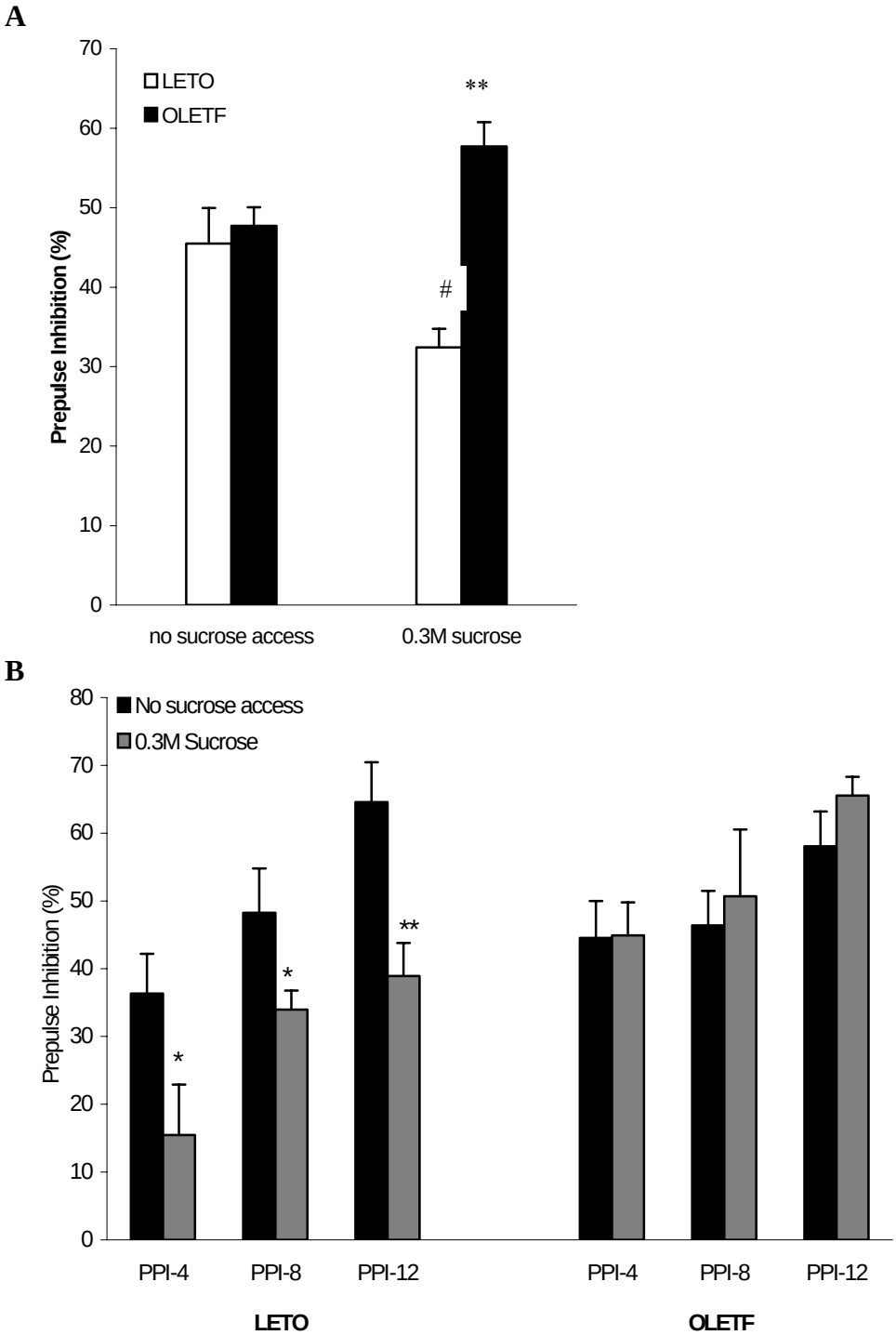


Figure 5.2: ALTO rats receiving sucrose treatment had a lower overall PPI percentage than OLETF rats, while non-sucrose receiving controls exhibited similar PPI percentages. Additionally, sucrose receiving LETO rats had lower PPI percentage than non-sucrose LETO rats. (**P<0.01, between strains; #P<0.05 within strain). **B**. When examining specific effects of prepulse intensity on PPI of baseline and sucrose-induced changes in PPI, LETO rats show decreased PPI at all levels of prepulse intensity, while OLETF rats are unresponsive. (**P<0.01, *P<0.05; between strains).

DISCUSSION

These results show that short repeated access to a palatable sucrose solution can disrupt PPI in non-mutant rodents. Furthermore, we demonstrated that using a sucrose access model, rats lacking CCK-1 receptors are insensitive to the PPI effects of sucrose across three levels of prepulse intensity compared with their non-mutant LETO controls. Additionally, the present data demonstrate that brief access to sucrose resulted in higher ASR in OLETF rats compared to LETO rats.

The specific mechanisms through which sucrose affects PPI are unknown, although several lines of evidence point to the activation of the mesolimbic DA system. For example, sucrose consumption produces a rise in NAcc DA (Hajnal and Norgren 2001). Furthermore, bilateral lesions in the pontine parabrachial nucleus (PBN), the second major relay in the gustatory pathway, attenuate sucrose-related DA responses in the NAcc, while similar lesions to the gustatory thalamus do not (Hajnal and Norgren 2005). This finding demonstrates a functional connection between gustatory information processing and accumbens DA, while also suggesting that these actions occur within the brainstem and/or limbic DA systems, rather than the thalamo-corticostriatal circuitry. Thus, it is likely that sucrose consumption could alter PPI via increased mesoaccumbens DA release. Evidence supporting this notion comes from studies using AMPH or cocaine challenge (Feifel et al. 2003). For example, OLETF rats show enhanced DA release and do not respond to drug-induced disruptions of PPI from AMPH or the NMDA receptor antagonist , dizocilpine (Feifel et al. 2001). Overall, these data indicate that OLETF rats may have abnormalities secondary to the inborn absence of CCK-1 receptors that may impair DA autoregulation. Therefore, it is possible that impaired sucrose-induced DA

signaling due to the lack of CCK-1 receptor in OLETF rats could account for the blocked sucrose PPI response in these animals. However, this hypothesis remains to be directly tested.

There are also data suggesting cross-sensitization effects of sucrose and AMPH. Rats previously sensitized to AMPH showed increased locomotor activity after brief sucrose access than rats that were prior treated with saline (Avena and Hoebel 2003). A similar cross-sensitization was also observed where rats receiving intermittent sucrose access exhibited higher subsequent locomotor activity by AMPH challenge, compared to rats that did not receive AMPH injections (Avena and Hoebel 2003). Additionally, rats having higher baseline consumption of sucrose show increased AMPH-induced dopamine overflow in the posterior nucleus accumbens relative to low sucrose feeders (Sills and Vaccarino 1996). This is consistent with our recent results demonstrating a greater sensitization to AMPH in OLETF compared to LETO rats after access to sucrose (Hajnal et al. 2004) and may explain our findings of an increased preference for sucrose in OLETF rats (De Jonghe et al. 2005).

We also showed that sucrose access resulted in increased ASR in OLETF, but not LETO rats. To our knowledge, this is the first observation of increased ASR using brief access to sucrose as a stimulus. The results are in overall agreement with previous data showing a higher magnitude of dopamine agonist-induced ASR in OLETF relative to LETO rats (Feifel et al. 2001). Our finding of a trend toward increased baseline ASR in OLETF compared to LETO rats parallel those by Feifel and colleagues, although we did not achieve statistical significance. This slight discrepancy in the data between the two

laboratories might be attributed to the relatively modest number of animals, and therefore decreased statistical power, used in the present design.

It is well known that ASR is a commonly used model of anxiety, where increased ASR signifies greater anxiogenic effect. Yamamoto and colleagues recently observed that OLETF rats exhibit higher anxious behaviors in an elevated plus-maze test relative to LETO controls (Yamamoto et al. 2000). Thus is it possible the higher anxiety may explain increased ASR in the OLETF rat. How this behavior would interact with sucrose access to promote a genotype-dependent increase in ASR is not known, however it is possible that altered sensitivity to the rewarding properties of sucrose may differentially affect the basal anxiety state in OLETF and LETO rats. In these studies, although baseline ASR in OLETF rats was somewhat higher than in LETO, baseline PPI was identical between strains, while sucrose treated OLETF rats showed both higher ASR and PPI relative to LETO rats. The different directional change in ASR and PPI measures in the LETO rats is not surprising since previous data also show that ASR and PPI can change independently. It is also worth noting the small number of animals used within each experiment group in the current work. The results reported here should be viewed as preliminary and interpreted with some caution until larger, more mechanistic studies are performed in order to clarify the specific substrates mediating the present observations.

Taken together, this study has identified repeated brief sucrose access as a stimulus model able to disrupt prepulse inhibition. While the exact mechanism for this effect is not yet known, it is likely that the mesolimbic dopamine system, that also mediates affective characteristics of palatable sucrose, is compromised in disruption of PPI by sucrose ingestion. In the sweet-preferring OLETF rats sucrose had no effect, that

is PPI was unaltered after sucrose access in these animals. Therefore, it is possible that developmental defects due to the congenital lack of CCK-1 receptor leads to alterations in DA signaling that becomes manifest by stimulation from sucrose ingestion which, in turn, leads to altered startle responsiveness. These changes, together with reported deficits in satiation signals of OLETF rats, may be responsible for the hyperphagia and obesity that characterize this model.

CHAPTER 6

HYPERSENSITIVITY TO THE INHIBITORY FEEDING EFFECTS OF DOPAMINE RECEPTOR ANTAGONISM IN THE PRE-DIABETIC OLETF RAT

INTRODUCTION

Potsingestive factors play an important role in the preference and intake of sweet taste preference (Sclafani and Nissenbaum 1987), however rats used in sham feeding experiments ingest sucrose solutions in a concentration dependent manner (Geary and Smith 1985). Such findings suggest that the orosensory experience associated with sucrose is sufficient to initiate and maintain ingestion. Our prior work shows a right-shifted sham feeding curve for sucrose intake in the OLETF rats relative to LETO controls. Data from Hajnal et al. using brief access lickometry also suggest enhanced sucrose taste sensitivity in the OLETF rat (Hajnal et al. 2005). Taken together, these findings may implicate possible alterations in food reward or sensory mechanisms to be partially responsible for such effects.

The mesoaccumbens DA system (MADS) has traditionally been implicated in not only the regulation of drug use behaviors, but in ingestive food reward-guided behaviors as well (Hoebel 1985; Hajnal and Norgren 2001; Sederholm et al. 2002; Rada et al. 2005). Low D2/D3 receptor binding in the MADS has been linked to higher rates of cocaine self-administration in primates (Morgan et al. 2002), while in humans, relatively diminished D2/D3 density has been observed in drug-dependent patients (Volkow et al.

1999). It has also been reported that obese humans show significantly decreased striatal D2/D3 receptor expression (Wang et al. 2001). Furthermore, the terminal regions of mesolimbic DAergic projections in the NAcc have been shown to be sensitive to dopamine receptor blockade in sucrose intake tests (Hajnal and Norgren 2001).

Data from Feifel et al. (Feifel et al. 2003), as well as from our own lab (De Jonghe et al. 2005), suggest altered DA signaling in OLETF relative to LETO rats. The Hajnal lab has also shown decreased DAT binding in the shell of the NAcc in the OLETF rat as further evidence of altered dopaminergic signaling in these animals (Hajnal et al. 2004). Of particular relevance are data linking DA involvement to food reward pathways come from studies using DA receptor antagonists for both D1/D5 (e.g. SCH23390) and D2/D3 (e.g. raclopride) receptor families. These data have clearly shown that intraperitoneal (IP) administration of these DA antagonists reduced sham-fed sucrose intake in a dose-dependent manner, which supports the notion that DA signaling facilitates or imparts a positive feedforward action on the orosensory effects of sucrose ingestion (Hsiao and Smith 1995; Smith 1995). The effects of these agents to reduce intake have been well established to occur within the brain, and not in the periphery (Baldo et al. 2002; Samson and Chappell 2003).

Therefore in order to identify DA dependency of altered preference for sucrose in OLETF rats, the current experiment was designed to investigate responses to dopamine receptor antagonism prior to ingestion of a palatable sucrose in OLETF and LETO rats .

METHODS

Subjects:

Thirty-six, male OLETF and LETO rats (423 ± 12.0 g and 335 ± 6.0 g for OLETF and LETO rats, respectively) were obtained as a generous gift of the Tokushima Research Institute, Otsuka Pharmaceutical, Tokushima, Japan. All rats were individually housed in mesh-floored, stainless-steel, hanging cages in a temperature-controlled vivarium while constantly maintained on a 12:12-hr light-dark cycle (lights on at 0600 hr, off at 1800 hr). Rats were handled daily for a minimum of one week prior to the onset of experimental procedures. Tap water and pelleted rat chow (Purina 5001) were available *ad libitum* throughout experiments except where indicated. All protocols used were approved by The Pennsylvania State University Institutional Animal Care and Use Committee.

Real feeding sucrose intake following dopamine receptor antagonism in OLETF and LETO rats.

In order to assess whether altered dopamine receptor sensitivity contributes to enhanced sucrose intake in OLETF rats, an equal number of age-matched OLETF and LETO rats were divided in 2 independent groups (n=5 per strain), one receiving D1/D5 antagonism, and the other receiving D2/D3 antagonism prior to sucrose intake (i.e. rats in D1/D5 antagonist group never received D2/D3 antagonists, and vice-versa). For these experiments, the D1/D5 antagonist SCH 23390 (SCH-23390 hydrochloride, FW-324.24, Sigma-Aldrich, St. Louis, MO) and the D2/D3 antagonist raclopride (s(−)-raclopride (+)-tartrate, FW-497.32, Sigma-Aldrich, St. Louis, MO) were used. Twelve week old rats were used for the current study over a period of 4 weeks. Corresponding body weights

were, at study start, 426 ± 15.0 g vs. 334 ± 6.0 g for OLETF and LETO rats, respectively; while at study completion, 489 ± 18.0 g vs. 380 ± 9.2 g, for OLETF and LETO rats, respectively.

Briefly, at 0800 hr each morning all rats had chow removed from home cages. At 0945 hr, water was removed and rats were intraperitoneally (IP) injected with either SCH23390 or raclopride. Fifteen minutes later, at 1000 hr, rats were then allowed 60 min ad libitum access to 0.3 M (10.26% (w/v)) sucrose solution via a calibrated glass drinking burette and recorded to the nearest 0.1 ml. Chow and water were returned following testing. Doses of receptor antagonist administered were: 50, 200, 400, 600, 800 nMol/kg: for both SCH23390 and raclopride. Drug doses were chosen based on previous studies showing inhibition of intake subsequent to IP administration of these drugs within these ranges (Weatherford et al. 1990; Hsiao and Smith 1995). Each dose was repeated a minimum of two occasions, with each drug day bracketed by a control, 0.9% saline injection day such that drugs were administered 48 hr apart.

Sham feeding of sucrose after pretreatment with D1/D5 and D2/D3 antagonists.

Rats were surgically implanted with gastric fistulae as described in Chapter 2. Our second study investigated the role of D1/D5 and D2/D3 receptors on the orosensory properties of sucrose ingestion using the sham feeding preparation. Twenty-three week old rats were used for the current study over a period of 4 weeks. Animal body weights for this experimental period were, at study start, 553 ± 13.2 g vs. 456 ± 6.1 g for OLETF and LETO rats, respectively; and upon study end, 569.3 ± 13.9 g vs. 441 ± 10.8 g, for OLETF and LETO rats, correspondingly.

At 1000 hr, following a brief 2 hr fast, the stainless steel screws occluding the gastric fistulae were removed, and stomach contents were lavaged with warm tap water to ensure minimal gastric volume upon start of sham feeding. Rats were placed into Plexiglas sham feeding boxes (8.5 x 29.0 x 30.5 mm: length, width, height) and acclimated to the sham feeding procedure by presenting them with 0.3 M sucrose for 60 min over several sessions until stable baseline intake was reached (~3-4 sham drinking sessions). Intakes were deemed stable when amount consumed on successive days varied by less than 10%. Rats were then grouped into independent D1/D5 or D2/D3 antagonist-receiving groups as in the previous experiment. All procedures of drug administration were identical to the previous experiment except that doses of antagonists administered were: 50, 200, 400, 800 nMol/kg for both SCH23390 and raclopride. Sham intake was measured to the nearest 0.1 ml every 5 min. In all sham feeding tests gastric drainage was collected in plastic graduated cylinders placed beneath the cages and the volume recorded at experiment termination. In the event that gastric drainage did not occur within 15 s of the start of sham feeding, the data from that subject were discarded for that specific sham feeding session on the basis that the gastric fistula was blocked, or not properly functioning (Yox and Ritter 1988).

Oral Glucose Tolerance Test (OGTT)

An oral glucose tolerance test was performed in a subset of rats (n=5, per strain) both before and after each set of real (10 and 17 weeks of age) and sham (20 and 27 weeks of age) feeding experiments. The test was administered following a 16hr fast, when an oral glucose load (2 g/kg) was delivered to each rat orally via latex gavage.

Blood glucose was measured before gavage and at 30, 60, 90, and 120 min post-glucose loading or insulin injection by a standard glucometer (LifeScan, One-Touch Basic). Animals were classified as diabetic if the peak level of plasma glucose was ≥300 mg/dl and a peak glucose level at 120 min > 200 mg/dl (Kawano et al. 1992) .

Statistical Analysis

Appropriate two-way repeated measures ANOVAs were performed with strain and drug dosage as main effects. Separate analyses were run for each drug administered. Percent suppression of sucrose intake from baseline due to dopamine receptor antagonism was calculated using the following formula: Percent Suppression = 1-(experimental/ baseline) X 100. For all experiments, ANOVA results were subsequently analyzed by Tukey's honestly significant difference (HSD) post-hoc tests when appropriate. All data were expressed as means $\pm$ SEM. Differences were considered statistically significant if $P<0.05$. Statistical analyses were computed with PC-SAS (version 8.02, SAS Institute, Carey, NC).

RESULTS

Suppression of real feeding sucrose intake following D1/D5 receptor antagonism in OLETF and LETO rats.

Figure 6.1A shows effects of SCH 23390 on suppression of real sucrose intake in OLETF and LETO rats. Results of ANOVAs showed a significant main effect of drug dose [$F(4,32)=31.0$, $P<0.001$], however no main effect for strain [$F(1,8)=1.8$; P=ns], or strain x drug dose interaction [$F(3.42)=0.3$, P=ns]. Thus, no significant differences in suppression of real sucrose intake were noted between OLETF and LETO rats following D1/D5 antagonism.

Suppression of real feeding sucrose intake following D2/D3 receptor antagonism in OLETF and LETO rats.

D2/D3 receptor antagonism effects on real sucrose intake in OLETF and LETO rats are illustrated in Figure 6.1B. Two way ANOVAs showed significant main effects of drug [$F(4,32)=41.3$, $P<0.0001$] and strain [$F(1,8)=11.6$; $P<0.01$] as well as a significant strain by x time interaction [$F(4,43)=5.8$, $P<0.01$]. In contrast to results after D1/D5 antagonism, post-hoc results demonstrate a greater suppression of real sucrose intake in OLETF rats compared to LETO rats at 600 (46.9 ± 8.8 % vs. 16.7 ± 10.7 % suppression for OLETF and LETO, respectively: $P<0.05$) and 800 nMol/kg doses (62.4 ± 8.8 % vs. 41.8 ± 4.5 % suppression for OLETF and LETO, respectively: $P<0.05$).

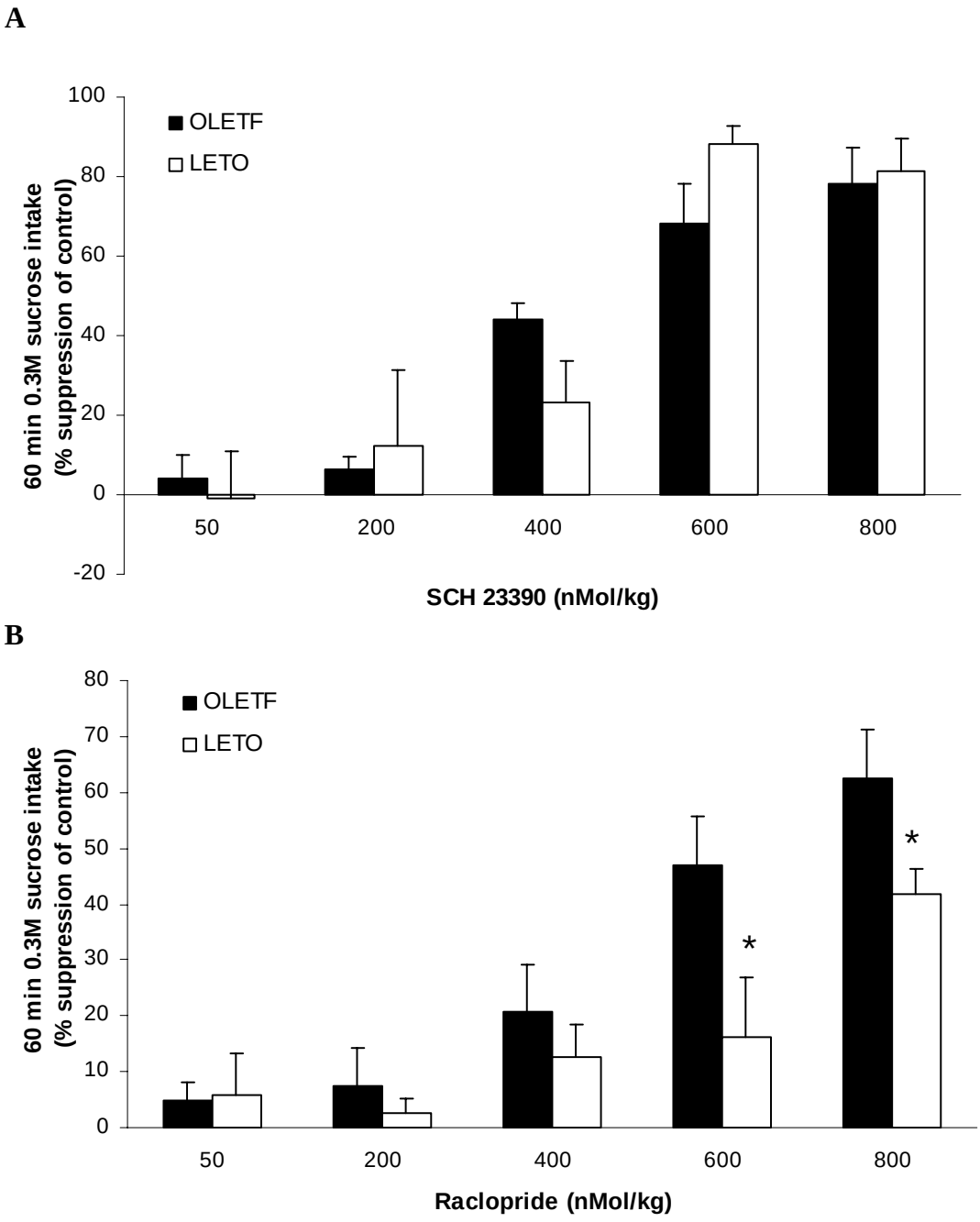


Figure 6.1 Suppression of 0.3 M sucrose intake in response to pre-treatment with the D1/D5 antagonist SCH 23390 and the D2/D3 antagonist Raclopride in real feeding OLETF and LETO rats. No differences between strain were noted after D1/D5 antagonism (A), while OLETF rats show a significantly greater suppression of sucrose intake at 600 and 800 nMol/kg doses of the D2/D3 antagonist raclopride (B). *P<0.05, between strains.

Suppression of sham feeding sucrose intake following D1/D5 receptor antagonism in OLETF and LETO rats.

Effects of SCH 23390 on sham sucrose intake in OLETF and LETO rats are depicted in Figure 6.2A. ANOVA results indicated significant main effect of drug dose [$F(3,42)=29.1$, $P<0.0001$], however no main effect for strain [$F(1,13)=1.1$; P=ns], or strain by drug dose interaction [$F(3.42)=0.1$, P=ns]. These results show that after D1/D5 antagonist SCH 23390 treatment, no significant differences in suppression of sucrose intake were noted between OLETF and LETO rats.

Suppression of sham feeding sucrose intake following D2/D3 receptor antagonism in OLETF and LETO rats.

Raclopride effects on sham sucrose intake in OLETF and LETO rats are illustrated in Figure 6.2B. ANOVA results showed significant main effects of drug [$F(3,39)=44.7$, $P<0.0001$] and strain [$F(1,13)=5.4$; $P<0.001$] as well as a significant strain by x time interaction [$F(3,39)=6.5$, $P<0.01$]. In contrast to results following D1/D5 antagonism, OLETF rats suppressed sham intake of sucrose to a significantly greater extent than LETO rats following D2/D3 antagonism. Post-hoc results revealed greater suppressions of intake in OLETF rats compared to LETO rats the two highest doses tested: 400 nMol/kg (38.7 ± 7.5 % vs. 12.9 ± 3.9 % suppression for OLETF and LETO, respectively: $P<0.01$), and 800 nMol/kg doses (67.0 ± 10.1 % vs. 30.3 ± 11.2 % suppression for OLETF and LETO, respectively: $P<0.05$).

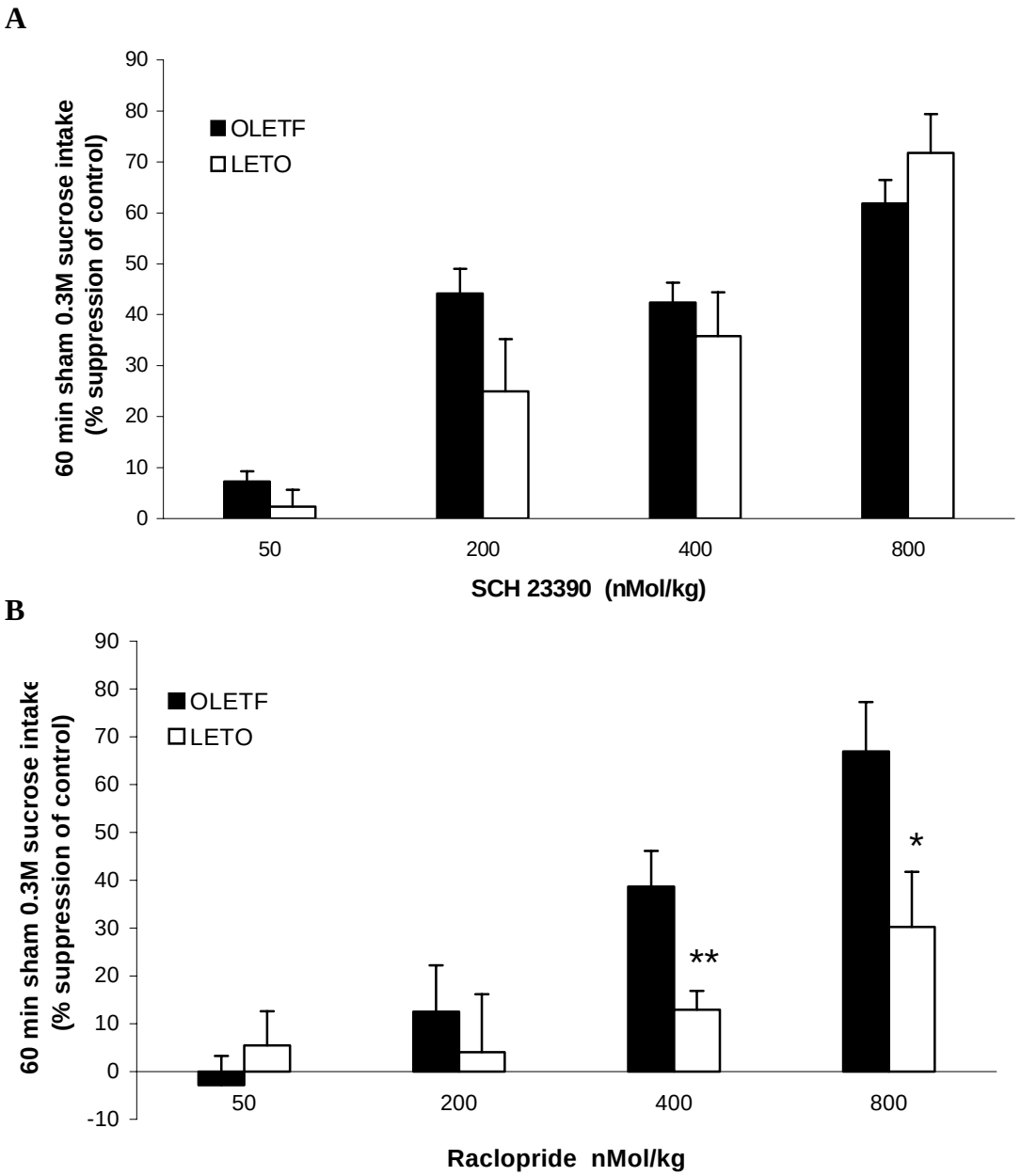


Figure 6.2 Suppression of 0.3 M sucrose sham intake in response to pre-treatment with the D1/D5 antagonist SCH 23390 and the D2/D3 antagonist Raclopride in real feeding OLETF and LETO rats. No differences between strain were noted after D1/D5 antagonism (**A**), while OLETF rats show a significantly greater suppression of sucrose intake at 600 and 800 nMol/kg doses of the D2/D3 antagonist raclopride (**B**). *P<0.05, **P<0.01, between strains.

Oral Glucose Tolerance Test

OLETF rats showed increased blood glucose levels relative to LETO rats in response to acute oral glucose challenge at all ages tested [Figure 6.3]. At 10 weeks old

[Figure 6.3A] and 17 wks old [Figure 6.3B], corresponding to animals ages before and after real feeding sucrose experiments, significant increases were observed in OLETF rats at 30 min and 60 min ($P<0.01$ for both time points) compared to LETO rats, with highest blood glucose peak at 30 min at both ages. Similarly, in 20 week old [Figure 6.3C] and 27 week old [Figure 6.3D] OLETF and LETO rats used in sham sucrose feeding studies, blood glucose measured post-glucose challenge were significantly higher in OLETF rats ($P<0.01$ for all time points), with highest blood glucose peaks again occurring at 30 min for both strains.

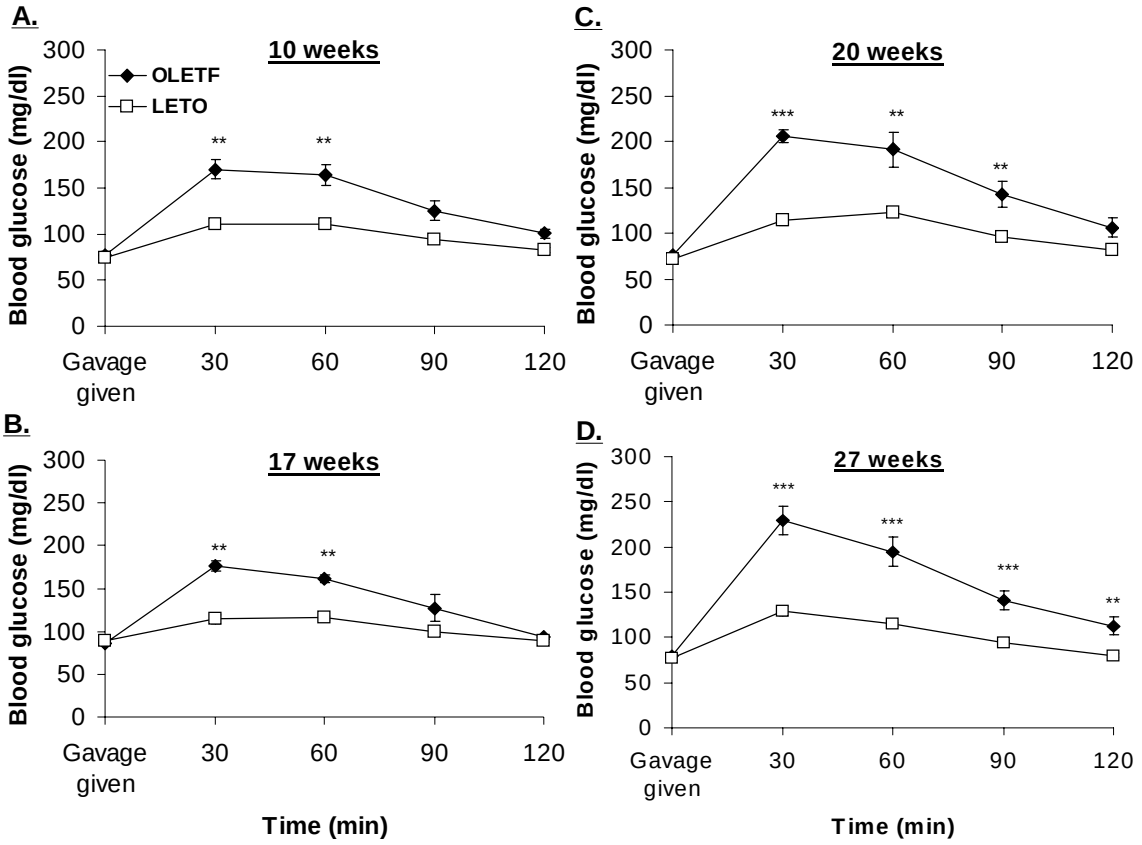


Figure 6.3: OLETF rats showed increased blood glucose levels relative to LETO rats in response to acute oral glucose challenge. At 10 weeks old [**A**] and 17 weeks old [**B**], corresponding to animals ages before and after real feeding sucrose experiments, significant increases were observed in OLETF rats at 30 min and 60 min ($P<0.01$ for both time points) compared to LETO rats, with highest blood glucose peak at 30 min at both ages. Similarly, in 20 week old [**C**] and 27 week old [**D**] OLETF and LETO rats used in sham sucrose feeding studies, blood glucose measured post-glucose challenge were significantly higher in OLETF rats ($P<0.01$ for all time points), with highest blood glucose peaks again occurring at 30 min for both strains. (**$P<0.01$,***$P<0.001$ for strain differences in blood glucose)

DISCUSSION

This experiment is the first to assess effects of DA receptor antagonists on sucrose intake in the OLETF rat. The findings show that in the presence or absence of post-gastric feedback, OLETF rats express an increased sensitivity to reductions in sucrose intake produced by DA receptor antagonism. These results demonstrate altered dopamine receptor sensitivity in obese OLETF rats with relevance to their increased sucrose preference.

DA receptor function has been closely linked to taste preference. For example, data from Hajnal and Norgren have shown that infusion of a D2/D3 receptor antagonist directly within the NAcc increased sucrose intake (Hajnal and Norgren 2001). This result was presumably due to the blockade of D2/D3 autoreceptors located on DA neuron terminals. Also of note in that study are the effects of low doses of simultaneously administered D1/D5 and D2/D3 antagonists to block increased NAcc DA release and sucrose intake. Additionally, increased DA release and sucrose intake due to administration of the DAT blocker nomifensine is also blunted after combined infusion of D1/D5 and D2/D3 antagonists (Hajnal and Norgren 2001). The findings of Hajnal and Norgren are important for two reasons: 1) they provide functional evidence of DA receptor involvement in regulating palatable sucrose intake and 2) the specific brain area (NAcc shell) reported is identical to that expressing the densest populations of CCK-1 receptors (Lanca et al. 1998). With regard to the latter, this co-localization of CCK-1 receptors and DA terminals may provide a basis for altered DA signaling in the OLETF rat. For example, DA and CCK are co-released in vivo after administration of drugs that increase neuronal firing rate, and furthermore CCK modulates DA release within the

NAcc by activating CCK-1 receptors (Crawley 1991; Ladurelle et al. 1994). Recent reports outside of this laboratory have suggested altered central DAergic signaling in the OLETF rat (Shilling and Feifel 2002; Feifel et al. 2003). Specifically, OLETF rats treated with DA agonists show both enhanced DA release (Feifel et al. 2003) as well as defective disruptions of prepulse inhibition response (Feifel et al. 2001). Increased DA release in response to DA agonism suggests a presynaptic, rather than postsynaptic, dysregulation of DA signaling contributing to a greater extracelluar DA response in the OLETF. This notion is consistent with the reported role of CCK-1 receptors in the autoinhibitory regulation of mesolimbic DA neurons after treatment with such agonists (Zhang et al. 1991). Therefore, in the absence of the CCK-1 receptor, OLETF rats consuming sucrose may achieve greater activation of mesoaccumbal DA neurons relative to LETO controls, which could lead to increased extracellular DA levels. Such an effect may be indicative of altered inhibitory D2 receptor activity on presynaptic terminals. Indeed, our effects with raclopride were observed at relatively high concentrations, suggesting alterations specific to autoreceptor populations. Thus, sucrose-evoked synaptic NAcc DA release in the OLETF may be higher compared to LETO rats, relative to a reduced baseline, due to the lack of tonic CCK-1 receptor stimulation.

It is also possible that OLETF rats show compounding effects of both pre- and post- synaptic alterations in DA signaling resulting from altered re-uptake and basal dopaminergic tone. In this context, our observations of similar sensitivities to D1/D5 antagonists do not suggest DA alterations in postsynaptic signaling, although our hypothesis of lower basal DA tone could suggest upregulation of D1/D5 receptors. The participation of altered D1/D5 mechanisms in sucrose intake in the OLETF rat, however,

cannot be discounted. It must also be mentioned that these data were collected using peripheral administration of D1/D5 and D2/D3 antagonists, which do not directly implicate the mesoaccumbens DA system in these effects. However, the current data do provide evidence of DAergic alterations in the OLETF rat in a model of natural reward known to affect mesoaccumbens DA signaling, complementing prior results using administration of DA agonists.

It is also possible that altered insulin activity in the OLETF rat contributed to these results. Interactions between CCK and insulin have been long reported (Riedy et al. 1995), and in addition, interactions between insulin and dopamine have also been studied. In fact, it has been suggested that insulin promotes dopamine output in the NAcc (Potter et al. 1999; Bello and Hajnal 2006). Additionally, insulin receptors are colocalized in dopamine cell bodies within the ventral tegmental area and substantia nigra (Figlewicz et al. 2003). Binding density of insulin in the NAcc is comparable to densities reported in the arcuate nucleus of the hypothalamus (Werther et al. 1987; Schulingkamp et al. 2000), suggesting not only a role for insulin in mediating body weight (Woods and Seeley 2001), but in food reward mechanisms as well. Results of OGTT analyses both pre- and post-experimentation do show evidence of hyperglycemia in the aging OLETF rat, yet not severe enough to constitute presence of NIDDM. However, given the known development of hyperglycemia and hyperinsulinemia due to insulin resistance in the OLETF rat across time, attention to insulin effects on dopaminergic signaling within the model is reasonable.

Since OLETF rats are also characterized by development of obesity, it is of interest that in other genetically obese animals, such as the ob/ob mouse, treatment with

D1/D5 and D2/D3 receptor agonists causes alterations in hyperphagia, hyperglycemia, hyperinsulinemia and elevations in body weight (Cincotta et al. 1997). Furthermore, D2/D3 receptor availability within the striatum of obese humans has been shown to be significantly lower, with BMI values inversely correlated with receptor availability (Wang et al. 2001). Thus, it is not unlikely that alterations in DA signaling in the OLETF could also contribute to impairments in body weight regulation.

Overall, these data indicate that OLETF rats may have abnormalities secondary to the inborn absence of CCK-1 receptors that may impair DA mechanisms. Therefore, it is possible that deficient sucrose-induced DA signaling due to the lack of CCK-1 receptor in OLETF rats may contribute to the overconsumption of sucrose in this strain.

Chapter 7

CORN OIL SHAM FEEDING AND RESPONSIVENESS TO DOPAMINE RECEPTOR ANTAGONISTS IN OLETF AND LETO RATS

INTRODUCTION

Rats exhibit strong preferences for foods high in fat (Levine et al. 2003). The associations governing these preferences, however, are due to both orosensory as well as postingestive consequences attributed to the food. In real feeding conditions, the volume of ingested fats or oils depends on both positive orosensory stimulating effects as well as postingestive nutritive feedback (Davis et al. 1995). Therefore, in this particular setting, the direct contributions of orosensory vs. postingestive components are not distinguishable. On the other hand, removing postingestive (or post-gastric) effects via sham feeing has led to findings that rats will sham feed corn oil emulsions in a concentration-dependent fashion (Mindell et al. 1990). These data support notions that the positive orosensory components of oils alone are sufficient to induce intake.

Like corn oil emulsions, sucrose solutions are also consumed in a concentration-dependant fashion when sham fed (Weingarten and Watson 1982). We have previously shown that OLETF rats exhibit altered sham intake of sucrose relative to LETO controls (De Jonghe et al. 2005). It is not known, however, whether OLETF rats have altered responsiveness to orosensory signaling of palatable solutions in general, or whether this behavior may be specific for solutions that taste sweet, such as sucrose. For these collective reasons, the first aim of this study was 1) to assess possible intake alterations in

the OLETF rat in response orosensory components of corn oil in the absence of post-gastric feedback, and 2) to explore the possibility of a generalized enhancement in orosensory sensitivity to preferred foods in the OLETF rat.

Central dopamine (DA) signaling has recently been implicated in not only the regulation of drug use behaviors, but in rewarding ingestive behaviors as well (Hajnal and Norgren 2001; Sederholm et al. 2002). It was recently reported that oral corn oil intake, but not intragastric infusion, is able to condition place preference (CPP), suggesting corn oil's orosensory effects to have rewarding effects (Imaizumi et al. 2000). Furthermore, the D1/D5 antagonist SCH 23390 was able to block corn-oil-induced CPP. Taken together, these results suggest that CPP was induced by the orosensory stimulating components of corn oil as well as mediated by D1/D5 receptor signaling. Additionally, intra-oral infusions of corn oil have been shown to enhance DA turnover rates within both the cortex and the midbrain (Sawano et al. 2000). Recent work also shows that rats sham-feeding oil exhibit an increase in NAcc dopamine similar to that of sucrose-feeding animals (Liang et al. 2006). Data using selective D1/D5 and D2/D3 receptor antagonists prior to sham intake of corn oil have reported dose-dependent reductions in corn oil intake (Weatherford et al. 1988). Administration of these drugs does not impair sham-water intake, nor do they induce obvious motor deficits. Thus, it has been suggested that D1/D5 and D2/D3 receptor antagonists reduce sham corn oil intake through disruptions of the orosensory and/or hedonic effects of corn oil (Weatherford et al. 1988).

Therefore, in order to examine the relative participation of dopaminergic receptor signaling in the orosensory controls of corn oil intake in OLETF and LETO rats, the

second aim of the current study was to assess effects of both D1/D5 and D2/D3 receptor blockade on sham corn oil intake.

METHODS

Subjects:

Twenty-two, male OLETF and LETO rats were obtained as a generous gift of the Tokushima Research Institute, Otsuka Pharmaceutical, Tokushima, Japan. All rats were individually housed in mesh-floored, stainless-steel, hanging cages in a temperature-controlled vivarium while constantly maintained on a 12:12-h light-dark cycle (lights on at 0600 hr, off at 1800 hr). Rats were handled daily for a minimum of one week prior to the onset of experimental procedures. Tap water and pelleted rat chow (Purina 5001) were available *ad libitum* throughout experiments except where indicated. All protocols used were approved by The Pennsylvania State University Institutional Animal Care and Use Committee.

Sham feeding of corn oil in OLETF and LETO Rats:

All rats were surgically implanted with gastric fistulae as described in chapter 2. After recovery from surgery, OLETF and LETO rats (n=5 per strain, weighing 493 ± 23.0 g and 384 ± 5.5 g for OLETF and LETO, respectively) were familiarized with the sham feeding cages and experimentation as described in Chapter 2. Corn oil was sham fed at 5 different concentrations: 12.5, 25, 50, 75, 100% (v/v) prepared with 0.75 ml Tween 80/100 ml corn oil. Pelleted food was continuously available in the home cages except during licking sessions. Rats were familiarized to the sham feeding first with

access to 12.5% corn oil, with presentation of oil across experimental days occurring in ascending concentration. Fluid intake was measured to the nearest 0.1 ml every 5 min over a 60-min feeding period following protocols identical to Chapter 2.

Sham feeding of 50% corn oil after pretreatment with D1/D5 and D2/D3 antagonists.

Naïve rats (n=6 per strain, weighing 408 ± 5.7 g and 320 ± 3.0 g for OLETF and LETO, respectively) were surgically implanted with gastric cannulae as described in chapter 2. Our first experiment showed a significantly increased intake of 50% corn oil in OLETF relative to LETO rats (see results section for complete analyses). To investigate whether this increased intake involved participation of D1/D5 and D2/D3 receptors, our second study investigated effects of selective dopamine receptor antagonists (SCH23390 and raclopride; D1/D5 and D2/D3 antagonists, respectively) on 50% corn oil intake. All procedures of drug administration, and sham feeding procedures were identical to those protocols described for dopamine antagonist sucrose sham feeding studies in Chapter 6, except that doses of antagonists administered were: 200, 400, 800 nMol/kg: for both SCH23390 and raclopride.

Statistical Analysis

For corn oil sham feeding experiments, appropriate two-way repeated measures ANOVAs were performed with strain and corn oil concentration as main effects. For DA antagonist studies, two way ANOVAs were run with strain and drug dosage as main effects. Separate analyses were run for D1/D5 and D2/D3 antagonist studies. Percent suppression of sucrose intake from baseline due to dopamine receptor antagonism was

calculated using the following formula: Percent Suppression = 1-(experimental/ baseline) X 100. For all experiments, ANOVA results were subsequently analyzed by Tukey's honestly significant difference (HSD) post-hoc tests when appropriate. All data were expressed as means $\pm$ SEM. Differences were considered statistically significant if $P<0.05$. Statistical analyses were computed with PC-SAS (version 8.02, SAS Institute, Carey, NC).

RESULTS

Sham feeding of various concentrations of corn oil in OLETF and LETO rats

Results of sham feeding various concentrations corn oil in OLETF and LETO rats are presented in Figure 7.1. Two way ANOVA results showed significant main effects of corn oil concentration [$F(5,40)= 4.4$; $P<0.01$] and strain [$F(1,8)=3.5$; $P<0.01$] however no significant interaction effect was noted (P=ns). Post-hoc analyses indicated 50% corn oil intake to be higher in OLETF rats relative to LETO rats (17.9 ± 3.1 ml vs. 8.4 ± 1.8 ml in OLETF and LETO rats, respectively; $P<0.05$). Although not statistically significant, a trend existed for increased intake of 12.5% (24.4 ± 7.4 ml vs. 8.4 ± 1.8 ml in OLETF and LETO rats, respectively; $P<0.12$) and 25% corn oil (17.9 ± 3.1 ml vs. 8.4 ± 1.8 ml in OLETF and LETO rats, respectively; $P<0.08$) in OLETF rats when compared to LETO rats. No significant differences in sham corn oil intake were noted between strains at all higher concentrations, including 100% corn oil without tween80 additive (as indicated by “100 pure” in figure 7.1).

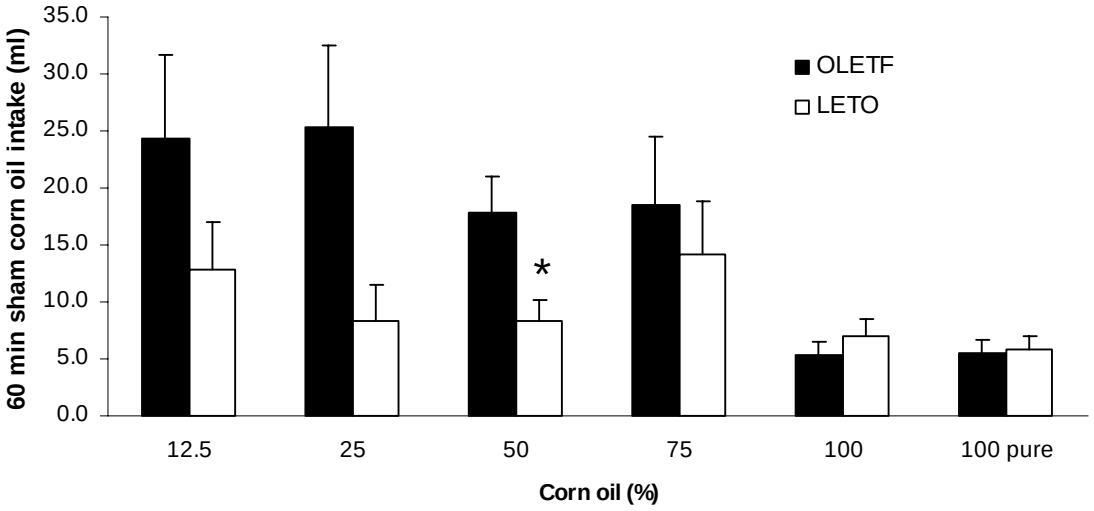


Figure 7.1: Corn oil sham intake in OLETF and LETO rats. 50% corn oil intake was significantly higher in OLETF rats relative to LETO rats, while no differences were noted at all other concentrations (*P<0.05 between strains)

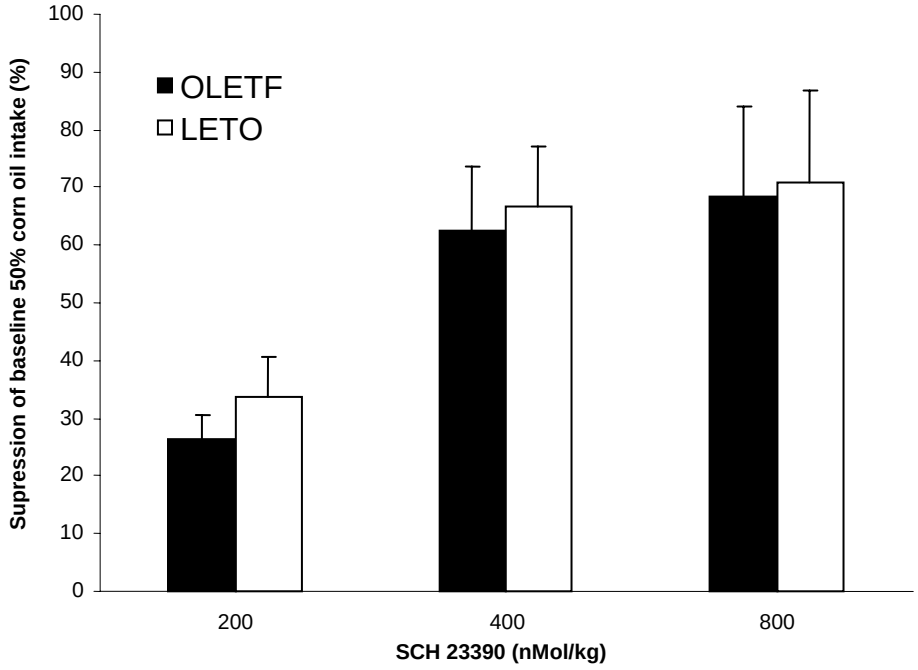
Suppression in sham feeding of 50% corn oil following D1/D5 receptor antagonism in OLETF and LETO rats.

Corn oil sham feeding data following D1/D5 antagonism were analyzed using percent suppression of baseline intake in OLETF and LETO rats (33.5 ± 9.9 vs. 8.7 ± 1.8 ml, in OLETF and LETO rats, respectively; P<0.01). Results of two way ANOVAs showed a significant main effect for SCH 23390 dosage [F(,2,18)=7.4, P<0.01], but no significant main effect of strain [F(1,9)=0.4,P=ns] or stain x drug dose interaction [F(2,18)=0.02, P=ns]. Suppression of sham corn oil intake following SCH 23390 pre-treatment was not significantly different between strains at either 200 (26.3 ± 4.2 vs. 33.8 ± 6.9 %, in OLETF and LETO rats, respectively), 400 (62.9 ± 11.0 vs. 66.8 ± 10.3 %l, in OLETF and LETO rats, respectively) or 800 (68.4 ± 15.6 vs. 70.8 ± 15.9 %, in OLETF and LETO rats, respectively) nMol/kg dosages.

Suppression in sham feeding of 50% corn oil following D2/D3 receptor antagonism in OLETF and LETO rats.

Sham feeding data in response to raclopride were analyzed using percent suppression of baseline corn oil intake in OLETF and LETO rats (37.1 ± 6.4 vs. 8.5 ± 2.3 ml, in OLETF and LETO rats, respectively; $P<0.01$). Two way ANOVA results revealed a significant main effect for raclopride dosage [$F(2,20)=8.0$, $P<0.01$], but no significant main effect of strain [$F(1,10)=0.8$,P=ns] or stain x drug dose interaction [$F(2,20)=0.2$, P=ns]. Sham corn oil intake suppression following raclopride administration at either 200 (15.0 ± 10.2 vs. 29.3 ± 18.2 %, in OLETF and LETO rats, respectively) , 400 (33.4 ± 9.0 vs. 50.1 ± 16.0 %, in OLETF and LETO rats, respectively) or 800 (71.0 ± 16.9 vs. 73.8 ± 9.3 %, in OLETF and LETO rats, respectively) nMol/kg dosages was not significantly different between OLETF and LETO rats.

A



B

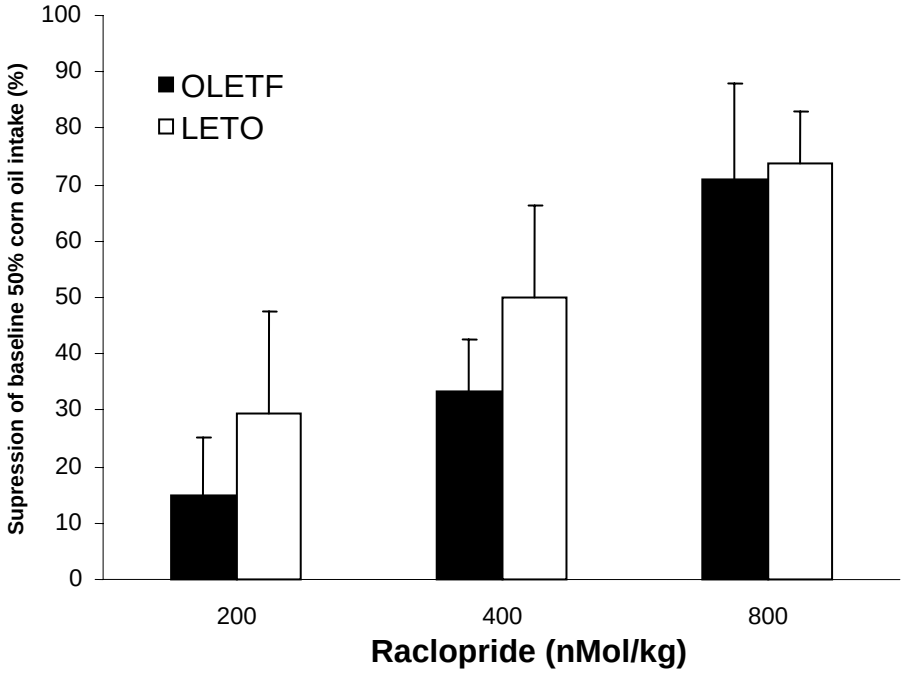


Figure 7.2 Suppression (%) of 50% corn oil intake following SCH 23390 (**A**) or raclopride (**B**) administration in OLETF and LETO rats. No significant differences in suppression of corn oil sham intake were noted between strains after D1/D5 or D2/D3 antagonism.

DISCUSSION

The present results show that OLETF rats exhibit altered orosensory responsiveness to corn oil. This effect appears most notably within relatively low concentrations of corn oil emulsions tested. It was also shown that disruption of dopamine signaling, via selective blockade of either D1/D5 or D2/D3 receptors, suppresses corn oil intake similarly between OLETF and LETO rats when only orosensory components of oil are experienced.

Orosensory responses to corn oil are very sensitive; corn oil emulsions with less than 1% corn oil content are sufficient to produce enhanced 1-bottle acceptance relative to plain water, as well as elicit distinctive preferences in 2-bottle choice tests (Mindell et al. 1990). It has traditionally been perceived that orosensory components of fat intake are related largely to tactile mechanisms associated with fat texture, mouthfeel or viscosity properties upon oral exposure (Mela 1988; Mindell et al. 1990). These data have suggested that responses by trigeminal systems may be the most likely sensory mechanisms responsible for discrimination of fat stimuli.

In the last few years there have been emerging implications of gustatory signaling involvement in the formation of preferences and intakes for fats (Tsuruta et al. 1999; Rice et al. 2000; Takeda et al. 2000). Most recently, Laugerette et al. have published results using the CD36-null mouse that implicate a role for the CD36 protein in the particular mediation of oral fat perception (Laugerette et al. 2005). Specifically, CD36 deficient mice did not express preferences for fatty acid enriched solutions. Although still controversial, these data are the first to support the presence of a functional fat taste receptor within the oral cavity. In light of these studies, it is possible that increased sham

corn oil intake in the OLETF may be due to altered trigeminal or gustatory processing of the orosensory stimulating effects of corn oil.

Evidence for altered gustatory signaling in the OLETF rat has been reported. It has recently been shown that sham feeding of sucrose is altered in the OLETF rat (De Jonghe et al. 2005). Furthermore, Hajnal et al. have reported that OLETF rats differ in gustatory functions with a generalized enhancement in sweet sensitivity (Hajnal et al. 2005). It is also noteworthy that a study by Tsunoda et al. demonstrated enhanced chorda tympani responses to high concentrations of sucrose (>1.0 M) in the OLETF rat (Tsunoda et al. 1998). Thus, at least in terms of sweet stimuli, it appears that OLETF show alterations in gustatory sensitivity. Furthermore, in the brief access tests reported by Hajnal et al., OLETF and LETO rats were also given capsaicin, a non-taste stimulus known to trigger trigeminal activity (Hajnal et al. 2005). Capsaicin lick rate was not significantly different between OLETF and LETO rats, suggesting that trigeminal sensory mechanisms in the OLETF rat may be intact. Taken together, enhanced corn oil sham intake in the OLETF may involve alterations in gustatory processing of the orosensory stimuli, further supporting prior data reporting such deficits in these animals. While trigeminal deficits in the OLETF rat do not appear evident from the current results, it is likely that corn oils activate trigeminal afferents (Mindell et al. 1990; Liang et al. 2006). It would be beneficial to assess effects of peripheral trigeminal sensory fiber activity on fat intake in OLETF and LETO rats to better elucidate trigeminal contributions to corn oil intake in the OLETF rat.

The current study also investigated effects of selective blockade of either D1/D5 or D2/D3 receptors in suppressing corn oil intake in OLETF and LETO rats, showing

similar suppressions of intake between strains. While no strain differences were reported, these results do suggest that DA receptor antagonism reduced the sensory and/or hedonic effects of orosensory components in the consumption of corn oil in OLETF and LETO rats. The current data are in contrast to findings assessing DA receptor blockade on sucrose intake, which showed that OLETF rats exhibit a hypersensitivity to D2/D3 receptor blockade in suppression of 0.3 M sucrose intake. Several possible explanations for a lack of parallel findings in corn oil and sucrose sham feeding data are likely. First, recent data have reported that transient increases in NAcc DA levels occur during sham intake of 100% corn oil (Liang et al. 2006) are similar in magnitude to 0.3 M sucrose (Hajnal and Norgren 2004). However, in our study, rats sham fed 50% corn oil. Thus, in the current study, the corn oil emulsion may have functioned as a weaker orosensory stimulus than pure corn oil and, in turn, could have potentially elicited a weaker DA response. The effects of multiple corn oil concentrations on DA output, as well as effects of DA receptor antagonists on ingestion, would be required to test this hypothesis. Second, corn oil and sucrose are inherently different stimuli, both in terms of intake patterns and sensory signal transduction. Namely, as discussed above, sucrose provides a largely gustatory excitatory response, while responses to fats and oils are highly controversial, and may involve both gustatory and trigeminal mechanisms (Mindell et al. 1990; Laugerette et al. 2005; Liang et al. 2006).

Finally, fats have been shown to affect opioid signaling within the NAcc (Will et al. 2003; Kelley et al. 2005). When given intermittent access to sugar solutions, rats exhibit increased binding of D1/D5 dopamine receptors and μ-1 opioid receptors in the nucleus accumbens (NAcc) (Colantuoni et al. 2001). Conversely, opioid antagonists

reduce intake of palatable foods in real (Cooper 1983; Cooper et al. 1985; Lynch 1986) and sham (Kirkham and Cooper 1988; Kirkham 1990) feeding, and decrease palatability in taste reactivity tests (Parker et al. 1992). It has also been shown that CCK-1 receptors may interact with opioid receptors or potentiate the actions of opioid agonists (Felicio et al. 2001; Quesada and Micevych 2006). Thus it is possible that altered or deficient opioid signaling could play a role in altered corn oil sham feeding in the CCK-1 receptor deficient OLETF rat.

In summary, OLETF rats exhibit altered intake of corn oil when post-ingestive effects were minimized. These strain differences may be attributed to altered gustatory processing within the OLETF rat, however alterations in trigeminal mechanisms cannot fully be discounted. DA receptors appear to participate in the reduction of sensory associations with the orosensory components of corn oil consumption similarly in OLETF and LETO rats, as effects of DA receptor blockade appear indistinguishable between strains. These latter findings contrast with results reported in sucrose intake, however it is possible that differences in the nature of orosensory stimulatory value, or alterations in other food reward-related substrate systems, may explain the distinctions between these findings.

CHAPTER 8

SUMMARY OF RESULTS, GENERAL DISCUSSION & PERSPECTIVES

Summary of Results

The spectrum of the current thesis work was designed to assess both gastrointestinal-derived inhibitory and orosensory stimulatory signals that could contribute to overconsumption in the OLETF rat. This goal was accomplished through examination of three specific aims.

Specific Aim 1: *Assess 1) alterations in solid and liquid gastric emptying rates in OLETF and LETO rats; 2) effects of volumetric gastric distension on sucrose sham feeding 3) vagal activation of hindbrain neurons following gastric distension in OLETF and LETO rat.*

As discussed in **Chapter 2,** despite showing increased food intake, OLETF rats do not express deficits in their ability to control gastric emptying across multiple levels of gastric capacitance, emptying periods and composition of gastric loads. In contrast, OLETF rats did show diminished response to gastric distension when sham feeding and when assessing vagal excitation via expression of Fos-LI nuclei. Thus, it is unlikely that hyperphagia in these animals involves abhorrent gastric emptying control; however, decreased gastric mechanosensation and detection of gastric volume within a meal, in combination with previously described satiation defects, may facilitate overconsumption through a lack of peripheral and neuronal negative feedback input.

Specific Aim 2: *Examine whether OLETF rats have increased sensitivity to the orosensory properties of sucrose or corn oil that may contribute to hyperphagic eating behavior.*

Experiments proposed within specific aim 2 were set forth in order to expand on data from the Covasa lab and others (Moran et al. 1998; Schwartz et al. 1999; Covasa and Ritter 2001; Bi and Moran 2002), as well as *Specific Aim 1*, by examining orosensory and positive feedback functioning in OLETF rats as a possible cause for hyperphagia, in addition to negative feedback deficits. **Chapter 3** presents results of the first investigations of taste preference functions in the OLETF rats. In two-bottle real-feeding preference tests, OLETF rats exhibited a greater preference for sucrose solutions compared to LETO rats. Furthermore, when orosensory components of sucrose were minimized via sham feeding preparations, OLETF rats sham fed more of concentrated sucrose solutions relative to LETO animals. To discern orosensory from post-gastric factors that may contribute to this preference, in a separate experiment, rats were allowed to sham feed sucrose in the absence or presence of duodenal sucrose infusion. Our results showed that intraduodenal sucrose infusions produced a smaller reduction of sucrose intake in OLETF vs. LETO rats, suggesting that altered inhibitory feedback signals of sucrose may also contribute to altered sucrose preference.

To assess whether this altered preference to palatable sucrose solutions reflected an overall increased orosensory sensitivity, the next experiment (**Chapter 7)** examined the potency of corn oil emulsions in an identical design using the sham feeding preparation. The results showed that under similar conditions that isolated orosensory

effects of the emulsion, OLETF rats exhibited increased consumed of oil emulsions. These findings demonstrate that OLETF rats display an overall increased oral preference for palatable ingestive stimuli. These strain differences may be attributed to altered gustatory processing within the OLETF rat, however alterations in trigeminal mechanisms are also possible.

Experiments reported in **Chapter 4** assessed how OLETF rats alter intake, preference, and conditioned preference of palatable solutions after acute food deprivation in order to examine how different motivational states impact these functions in the OLETF rat. Our results showed that after chow restriction LETO rats both increased both sucrose intake and two-bottle sucrose preference relative to their free-fed baseline, whereas OLETF rats did not. In contrast, OLETF rats exhibited a higher conditioned flavor preference when sucrose or saccharin was used as an unconditioned stimulus in conditioned preference studies. These findings suggest that OLETF rats show a greater influence of orosensory effects of sweet solutions when forming associations of those foods, relative to LETO controls.

Thus, the results comprising *Specific Aim 2* suggest that OLETF rats form preferences for sucrose based largely on orosensory and hedonic properties of the solution, rather than post-ingestive associations. The enhanced responsiveness to oral stimulation, coupled with deficits in responding to the postingestive feedback of intestinal sucrose, may contribute additively to the development of hyperphagia and weight gain in OLETF rats.

Specific Aim 3: *Explore the role of the dopaminergic system as a possible substrate contributing to enhanced preference for and intake of palatable foods in OLETF rats.*

In addition to its role in peripheral satiation signaling, CCK found within specific brain regions plays a modulatory role in the activity of many neurotransmitters, including dopamine (DA) (Crawley 1991). The effects of oral sucrose on dopaminergic transmission are well recognized. In **Chapter 5**, it is reported that repeated brief sucrose access as a stimulus, is able to disrupt prepulse inhibition. While the exact mechanism for this effect is not known, it is likely that the mesolimbic dopamine system, that also mediates affective characteristics of palatable sucrose, is compromised in disruption of PPI by sucrose ingestion. In OLETF rats, sucrose had no effect, that is PPI was unaltered after sucrose access in these animals. Therefore, it is possible that developmental defects due to the congenital lack of CCK-1 receptor leads to alterations in DA signaling that becomes manifest by stimulation from sucrose ingestion which, in turn, leads to altered startle responsiveness.

Chapter 3 demonstrated that OLETF rats have an increased preference for both real and sham feeding of sucrose solutions. Sham feeding sucrose in rats triggers NAcc DA release as a function of sucrose concentration (Hajnal et al. 2004). Furthermore, studies using DA receptor antagonists for both D1/D5 and D2/D3 receptor families have shown that these receptors are necessary for normal central processing of orosensory effects of palatable solutions (Weatherford et al. 1990). To more directly examine altered DA signaling in the OLETF rat, **Chapter 6** presents results that OLETF rats express a

hypersensitivity to dopamine receptor antagonists, of both D1/D5 and D2/D3 receptor families in both real and sham feeding conditions. Results of DA receptor antagonism following corn oil sham intake in **Chapter 7** show that this hypersensitivity to DA receptor antagonism may not extend to corn oil tastants.

General discussion and perspectives

Knock-out and spontaneous mutant rodent models have been used in investigation of regulation of feeding in general, and neural mechanisms underlying the development of dietary-induced obesity in particular. Among these is the CCK-1 deficient OLETF rat, a spontaneous CCK-1 receptor knockout strain. This rat provides a unique model for the study of signals mediated by CCK-1 receptors in control of food intake. The role of CCK-1 receptors, which are located predominantly in the periphery, in the control of food intake and gastrointestinal functions is well established (Ritter et al. 1999). Therefore, until recently, this model has been used almost exclusively for studying a potential peripheral domain of food intake regulation. Using OLETF rats, several studies, including our own (Covasa and Ritter 2001; Covasa and Ritter 2005), provided compelling evidence of CCK-1 receptor participation in control of food intake (Bi and Moran 2002). Previous work in intact animals has shown that CCK-1 receptors do not participate in long term control of food intake, since overall food intake remains unchanged following exogenous administration of CCK (Crawley and Beinfeld 1983; West et al. 1984). However, CCK-1 receptor deficient rats are hyperphagic and become obese, suggesting two possibilities: 1) the absence of the CCK-1 receptor leads to

disordered energy balance, 2) other non-CCK related defects, or secondary to the lack of CCK-1 receptor, may exist in the OLETF rat which regulate feeding and body weight.

There is emerging support for both hypotheses. Related to the first point, several studies have observed interactions between CCK and leptin signaling in the controls of food intake and body weight (Barrachina et al. 1997; Matson et al. 2000; Matson et al. 2002). OLETF rats are hyperleptinemic, attributed due to increased adipose accumulation concomitant to obesity development (Niimi et al. 1999). It has been shown that pair-feeding OLETF rats to intakes of LETO rats ameliorates both increases in weight gain as well as hyperleptinemia, suggesting leptin deficits in the OLETF rat are secondary to hyperphagia and obesity (Bi et al. 2001). Indeed, centrally administered leptin reduces food intake similarly in OLETF and LETO rats (Niimi et al. 1999). Therefore, the absence of CCK-1 receptors in the OLETF rat, and thus also deficient CCK effects on leptin signaling, may result in a down-regulation of leptin signaling that may contribute to both hyperphagia and obesity in this strain.

Deficits in the OLETF rat secondary to the CCK-1 receptor have also been suggested. Prior work has shown not only shown CCK-mediated defects in satiation in the OLETF (Moran et al. 1998; Covasa and Ritter 2005), but also to nutrient-induced satiation controls (Covasa and Ritter 2001). Our own assessments of gastric function in the OLETF have provided evidence supporting preserved gastric emptying: a physiological mechanism mediated by CCK-1 receptors. However, our results suggest diminished gastric mechanodetection of volume: a process in which recent investigations have provided data excluding CCK-1 receptor activation as playing a role in the vagal induction of hindbrain c-Fos following gastric distension (van de Wall et al. 2005).

Clearly, these collective results support speculations of secondary abnormalities in the OLETF exist beyond the lack of CCK-1 receptor expression.

The studies presented here have also explored potential central dopamine mechanisms associated with motivational and food reward behaviors that may interfere with processing of critical information in control of food intake in the OLETF rat. These results are the first to show altered motivational and sensory processing of orosensory stimuli of palatable foods that may contribute to hyperphagia in the OLETF rat. Of particular importance are the current results showing increased sucrose and corn oil intake and preference, as they suggest enhanced gustatory sensitivities to palatable orosensory stimuli in the OLETF rat. Alterations in dopaminergic signaling have been implicated as a contributor to this altered orosensory control in sucrose intake. Our observations of enhanced suppression of sucrose intake following D2/D3 receptor antagonism suggest that the central processing of the orosensory effects of sucrose is altered in the OLETF rat. The neuroanatomical and functional evidence linking CCK-1 receptor activation to stimulation of dopamine release in non-mutant animals is abundant (Hill et al. 1990; Crawley 1991; Hamilton and Freeman 1995). Data presented here, as well as others, provide further support in demonstrating the involvement of altered dopamine signaling in the consumption of palatable sucrose in the CCK-1 deficient OLETF rat (Feifel et al. 2001; Feifel et al. 2003; Hajnal et al. 2004). Although the exact central mechanisms behind our effects of dopamine alterations and sucrose intake remain unclear, the observed deficits in OLETF rats may be due to the absence of CCK-1 receptor mediated effects on either specific taste receptors encoding for sweet stimuli, or within gustatory relays to the NTS and/or projections to the gustatory centers of the

parabrachial nucleus (PBN). Nonetheless, the possible existence of food reward deficits proposed here suggest that OLETF rats provide a largely untapped in vivo model to test the role of the CCK-1 receptor in alterations of food reward mechanisms leading to disordered eating.

The data herein of course are not without shortcomings. First, these data primarily focus on sucrose solutions as the orosensory stimuli. Transient increases in DA levels can occur after ingestion of meals consisting of multiple macronutrient sources and deprivation conditions (Wilson et al. 1995; Martel and Fantino 1996; Hajnal et al. 2004; Liang et al. 2006). A more detailed analysis of orosensory sensitivities in the OLETF to multiple tastants is warranted, especially given the evidence for altered licking rates of several distinctive sweet solutions, including sucrose, while bitter and sour tastants were unaltered (Hajnal et al. 2005). Along these lines, the examinations at hand of altered dopaminergic signaling involved only one preferred concentration of sucrose. While the data here are compelling, it is nonetheless necessary to explore multiple ranges of sucrose volumes, concentrations, and methods of oral delivery (i.e., free-feeding vs. oral catheters) in order to more conclusively address and control for overall orosensory stimulus strength and rate of oral delivery, which could potentially confound or mask results. Second, the possible role of insulin activity in these results cannot be discounted. Interactions between CCK and insulin have been long reported (Riedy et al. 1995), and in addition, interactions between insulin and dopamine have also been studied. In fact, available data suggest that insulin promotes dopamine output in the NAcc (Potter et al. 1999; Bello and Hajnal 2006), while insulin receptors are colocalized in dopamine cell bodies within the ventral tegmental area and substantia nigra (Figlewicz et al. 2003).

Binding density of insulin in the NAcc is comparable to densities reported in the arcuate nucleus of the hypothalamus (Werther et al. 1987; Schulingkamp et al. 2000), suggesting not only a role for insulin in mediating body weight (Woods and Seeley 2001), but in food reward mechanisms as well. Indeed, brief access taste responsiveness in the OLETF to sweet stimuli were altered as a function of age, an effect paralleled by the development of hyperglycemia (Hajnal et al. 2005). Given the development of hyperglycemia and hyperinsulinemia in the OLETF rat, attention to insulin effects on dopaminergic signaling within the strain is reasonable. Third, the site of action of the pharmacological results reported here is not known. While considerable speculation and attention has been devoted to the NAcc region, secondary or reciprocal DA projections to and from other brain areas cannot be disregarded, such as the prefrontal cortex or central nucleus of the amygdala, among other possible sites of action. It is possible that dopaminergic alterations exist in multiple regions of the OLETF brain, not just those directly mediated by CCK-1 receptor activation. It is also necessary to discern within the D2/D3 family, whether or not our reported efforts involve D2/D3/D3 autoreceptor populations, post-synaptic sites, or both. Reverse and no-net flux microdialysis experiments infusing dopamine receptor antagonists directly within these areas to measure DA output and metabolites would no doubt help to elucidate some of these issues. Finally, it must also be acknowledged that a significant body of work implicates the potent central orexigen NPY in hyperphagia in the OLETF rat. Specifically, increased NPY in the DMH has been cited as a possible cause of increased appetitive behavior in the OLETF rat by stimulating ingestion (Bi et al. 2001; Bi and Moran 2002; Bi et al. 2005). While altered signaling of this neurotransmitter no doubt contributes to increased food intake, very recent data

suggest that altered DMH NPY activity in the OLETF rat does not play a role in increased motivation for sucrose consumption specifically (Azzara et al. 2006), and thus may not play a role specific to the altered orosensory control of palatable food intake.

Within these limitations, our results show that OLETF rats exhibit increased orosensory sensitivities leading to enhanced intake of palatable solutions, an effect mediated through altered dopaminergic signaling. These results suggest a model of dopaminergic activity in the OLETF rat of both decreased dopaminergic tone (i.e., lower basal level of dopamine output), in addition to exaggerated synaptic dopamine release in response to oral sucrose. In such a model, the feed-forward relationship between sucrose ingestion of dopamine output would be much greater in the OLETF, which could be exemplified by the much higher degree of hyperphagia in response to highly palatable foods relative to LETO controls.

The shell region of the NAcc has been implicated as the most likely site of action for this proposed mechanism. Indeed, this model fits well with other data reported in the OLETF rat outside of ingestive behavior (Feifel et al. 2001; Feifel et al. 2003). As these animals also develop multiple phenotypic and metabolic derangements throughout development, it can be expected that alterations in dopaminergic pathways become increasingly more marked as the animal ages: further exacerbating and promulgating overconsumption and body weight gain in the OLETF rat.

When regarding this research in a wider scope, these studies impact the functional relevance of the CCK-1 receptor in mediating positive feedback mechanisms governing food intake. This receptor functions not just as a mediator of peripheral satiation controls of food intake, but also may play an important role in potential positive/stimulatory

influences on hyperphagia leading to body weight gain and obesity. It is also important to be aware of emerging data suggesting dopaminergic alterations in both the development of obesity, as well as disordered eating (Wang et al. 2002). In fact, recent human data suggest that dopamine receptor availability is inversely correlated with body mass index (Wang et al. 2001), suggesting that the development of obesity may be paralleled by deficits in dopaminergic function. The current human obesity epidemic is not likely due to genetic causes, however it is increasingly clear that systems regulating caloric intake and energy balance communicate with systems of food reward response. In actuality, it is entirely possible and probable that neurotransmitters and hormones, such as insulin and leptin, thought to be primarily involved in body weight regulation within the hypothalamus, may directly act on dopaminergic neurons to mediate responses to food reward (Figlewicz et al. 2003). The current work supports these integrative notions, as the OLETF rat is an animal characterized by deficiencies in apparent short-term satiation signaling, yet also exhibits accelerated weight gain, as well as altered sensitivity to the rewarding properties of palatable foods.

Potentially, further investigations in the OLETF may clarify the crosstalk between these two complementary inhibitory and stimulatory systems by teasing apart to roles of CCK and dopamine in an *in vivo* model where both systems are impaired. As the data across disciplines continue to merge, it will become more and more necessary to consider models such as this (i.e. where multiple pathways and neurotransmitters are affected) in order to fully appreciate the feedback circuitry systems governing food intake, reward, and ultimately body weight regulation.

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3. **Bart C. De Jonghe,** Andras Hajnal, and Mihai Covasa. Decreased gastric mechanodetection, but preserved gastric emptying, in CCK-1 receptor deficient OLETF rats. Am J Physiol Gastrointest Liver Physiol. *Epub 2006 May 25.*

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