GLUCAGON-LIKE PEPTIDE-1 MEDIATES
HEDONIC INTAKE AND FOOD REWARD

A Dissertation in
Neuroscience
by
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ABSTRACT

Current theories on the cause and prevention of obesity highlight the importance of the reward system in feeding, food choices, and hyperphagia. Palatable foods, commonly high in fat and sugar content, can engage the reward system and increase motivation to procure and consume such foods, independent of caloric need or nutritional value. Excessive consumption of highly palatable foods is believed to be a key environmental factor in obesity rates. Of major concern are the complex and long-lasting changes to various neural systems following obesity. Among these include changes to mesolimbic dopamine signaling along the central reward pathway; altered dopamine signaling has been linked with both a genetic predisposition for obesity and dietary induced obesity.

Recently, more attention has been given to alterations in the gut-brain system that occur with obesity, such as notable differences in the incretin hormone glucagon-like-peptide-1 (GLP-1). Interestingly, obesity-induced GLP-1 deficits appear to be reversed following Roux-en-Y gastric bypass surgery, and changes to GLP-1 and similar peptides have been theorized as critical factors in the success of this surgical procedure. While GLP-1 has been clearly defined as both an incretin and a satiety hormone, less is known about the influence of GLP-1 on systems driving hedonic intake and food reward. Furthermore, the debate continues as to the role of GLP-1 in obesity, and its precise impact in Roux-en-Y gastric bypass surgery.

To investigate the role of GLP-1 on the dopamine reward system, and how GLP-1 is influenced in obesity, we investigated how temporarily altering the dopamine and GLP-1 systems of lean and dietary induced obese (DIO) rats influenced hedonic intake of palatable solutions using the synthetic GLP-1 agonist Exendin-4 and receptor specific dopamine antagonists. Peripherally administered dopamine antagonists led to significant reductions in the intake of palatable carbohydrates, with notable differences between the two dietary obese groups despite similar body weight gain; one group was fed a high fat-high energy diet and one fed a fat-carbohydrate high energy combination diet. Furthermore, these differences were dependent upon the receptor subtype targeted, with D1 receptor antagonism producing more potent reductions than D2 receptor antagonism, and
upon the type of carbohydrate, with sucrose intake more susceptible to dopamine antagonism than fructose intake. These data indicate dopamine signals do change in obesity, and the type of diet leading to obesity can influence the extent and perhaps nature of such changes. This data implies that differential responses may occur to different palatable foods, and indicates why individuals may respond to different treatment regimens with various success rates.

Using an identical animal model of two DIO groups, we again compared responses to sucrose and fructose in chow-fed lean and DIO rats after activation of the GLP-1 receptor by the synthetic analog Exendin-4. Exendin-4 (ip) reduced both sucrose and fructose intake, and the magnitude of the effect varied with respect to the obesogenic diet and the type of carbohydrate being tested. As in the dopamine receptor inhibition tests, all groups reduced sucrose intake following treatment, but the reduction in carbohydrate intake, particularly to fructose, was attenuated in animals made obese on the high-fat compared to the fat-carbohydrate combination diet.

Upon establishing similar effects to dopamine receptor antagonism and GLP-1 receptor activation peripherally, we then explored the central GLP-1 system, localizing the effects of GLP-1 on both hedonic intake and food-reward. After establishing a role for GLP-1 within the ventral tegmental area (VTA) using behavioral methods, we then examined the anatomical and neural relationship between GLP-1 and the dopamine system using immunohistochemistry and electrophysiology. Our findings are the first to directly demonstrate that GLP-1 in the VTA is not only behaviorally important, altering both hedonic intake and the motivation to procure sucrose, but may also have powerful influence on the dopaminergic system through the presence of GLP-1 receptors throughout the VTA. GLP-1 receptors were not only found on VTA dopaminergic neurons, but microiontophoretic application of GLP-1 directly upon dopamine neurons also resulted in significantly altered firing. These novel findings represent a powerful new potential role for GLP-1 in the control of intake unrelated to hunger.

Finally, using an animal model of Roux-en-Y gastric bypass surgery, we investigated whether differences would occur between surgical subjects and their dietary obese Sham-operated
counterparts in their willingness to work for a sucrose reward, and how GLP-1 receptor activation changed operant responses. When administered peripherally, the long-lasting GLP-1 agonist Exendin-4 did not alter behaviors. When applied centrally, Exendin-4 attenuated the willingness of GBS and Sham rats to work for and consume a sucrose reward, and the GLP-1 receptor antagonist Exendin-9 blocked this effect. Furthermore, when looking at levels of GLP-1 receptor mRNA within the midbrain in our surgical and sham cohorts compared to naïve chow-fed lean controls, we found when comparing Roux-en-Y and Sham animals to our chow controls, the surgical procedure seemed to return receptor levels to a state more congruent with that observed in lean controls.

Taken together, these data clearly demonstrate a role for GLP-1 in the control of intake related to palatable, hedonic foods. We described how activation of the peripheral GLP-1 system influences hedonic intake of palatable carbohydrates in a manner similar to blocking peripheral dopamine signaling. Furthermore, we found that central GLP-1 may play a particularly important role in the seeking and consumption of highly palatable foods by acting directly on the mesolimbic dopamine system. These findings indicate GLP-1 may have further clinical potential in reducing hedonic appetite and food cravings.
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<tr>
<td>$^1$H-NMR</td>
<td>Proton-Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>4V</td>
<td>Fourth (IV) Ventricle</td>
</tr>
<tr>
<td>aCSF</td>
<td>Artificial Cerebral Spinal Fluid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>ARC</td>
<td>Arcuate Nucleus of the Hypothalamus</td>
</tr>
<tr>
<td>AP</td>
<td>Anterior-Posterior (coordinates from Bregma)</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-Brain Barrier</td>
</tr>
<tr>
<td>BCC</td>
<td>Bicuculline Methiodide, selective GABA&lt;sub&gt;A&lt;/sub&gt; receptor antagonist</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>BW</td>
<td>Body Weight</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic Adenosine Monophosphate</td>
</tr>
<tr>
<td>CCK</td>
<td>Cholecystokinin</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebral Spinal Fluid</td>
</tr>
<tr>
<td>D1R</td>
<td>Dopamine D1-like receptors</td>
</tr>
<tr>
<td>D2R</td>
<td>Dopamine D2-like receptors</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>dB</td>
<td>Decibels</td>
</tr>
<tr>
<td>DIO</td>
<td>Dietary Induced Obesity</td>
</tr>
<tr>
<td>DM</td>
<td>Diabetes Mellitus</td>
</tr>
<tr>
<td>DPP-IV</td>
<td>Dipeptidyl-peptidase-4</td>
</tr>
<tr>
<td>DV</td>
<td>Dorsal-Ventral (coordinates from Bregma)</td>
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<tr>
<td>Ex-4</td>
<td>Exendin-4, synthetic GLP-1 receptor agonist</td>
</tr>
<tr>
<td>Ex-9</td>
<td>Exendin-3 (9-39) amide, selective GLP-1 receptor antagonist</td>
</tr>
<tr>
<td>FCHE</td>
<td>Fat-Carbohydrate High Energy Diet</td>
</tr>
<tr>
<td>FI</td>
<td>Food Intake</td>
</tr>
<tr>
<td>FR</td>
<td>Fixed Ratio Schedule of Reinforcement</td>
</tr>
<tr>
<td>GABA</td>
<td>$\gamma$-Aminobutyric acid</td>
</tr>
<tr>
<td>GAD</td>
<td>Glutamate Decarboxylase (or Glutamic Acid Decarboxylase)</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>GIP</td>
<td>Gastric Inhibitory Peptide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
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<tr>
<td>GLP-1</td>
<td>Glucagon-like Peptide-1</td>
</tr>
<tr>
<td>GLP-1R</td>
<td>Glucagon-like Peptide-1 Receptor</td>
</tr>
<tr>
<td>Glu</td>
<td>L-glutamic acid (Glutamate)</td>
</tr>
<tr>
<td>HF</td>
<td>High Fat</td>
</tr>
<tr>
<td>HFCS</td>
<td>High-Fructose Corn Syrup</td>
</tr>
<tr>
<td>HFHE</td>
<td>High-Fat High Energy Diet</td>
</tr>
<tr>
<td>ICV (i.c.v.)</td>
<td>Intracerebroventricular</td>
</tr>
<tr>
<td>ID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Inhibitory Dose Required to Reduce to 50% of Baseline</td>
</tr>
<tr>
<td>IP (i.p.)</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>kcal</td>
<td>Kilocalorie(s)</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LV</td>
<td>Lateral Ventricle</td>
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<tr>
<td>mRNA</td>
<td>messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>ML</td>
<td>Medial-Lateral (coordinates from Bregma)</td>
</tr>
<tr>
<td>NAcc</td>
<td>Nucleus Accumbens</td>
</tr>
<tr>
<td>NS</td>
<td>Not Significant</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffer Saline</td>
</tr>
<tr>
<td>PC</td>
<td>Proconvertase</td>
</tr>
<tr>
<td>PSB</td>
<td>Pontamine Sky Blue</td>
</tr>
<tr>
<td>PR10</td>
<td>Progressive Ratio-10 Schedule of Reinforcement</td>
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<td>PVN</td>
<td>Paraventricular Nucleus of the Hypothalamus</td>
</tr>
<tr>
<td>PYY</td>
<td>Peptide YY</td>
</tr>
<tr>
<td>QP</td>
<td>Quinpirole, selective D2R agonist</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>RYGB</td>
<td>Roux-en-Y Gastric Bypass Surgery</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative Real-Time Polymerase Chain Reaction</td>
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<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SEM (s.e.m.)</td>
<td>Standard Error of Mean</td>
</tr>
<tr>
<td>T1R3</td>
<td>Taste receptor T1R family member 3</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine Hydroxylase</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral Tegmental Area</td>
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</table>
Acknowledgments

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Chapter 1.
Introduction

1.1. Neurobiology of Feeding, Hedonic Intake, and Food Reward

Years of research dedicated to understanding the processes engaged during typical, meal-related feeding behaviors led to the understanding that the central nervous system (CNS) is the primary controlling source of hunger, appetite, and satiety (Guyenet and Schwartz, 2012; Murphy and Bloom, 2004; Smith, 2000). Signals that originate not only from the gut and other peripheral systems, but also from nuclei within the CNS, act upon neurons in the hypothalamus to determine changes in appetite (Grill, 2006; Smith, 2000; Suzuki et al., 2010). These signals include a variety of neuroendocrine hormones released from the gut, such as peptide YY, oxyntomodulin, and glucagon-like peptide-1, of which the active form is commonly referred to as GLP-1 (Huda et al., 2006; Murphy and Bloom, 2004). Hormones from other cells, such as leptin release from adipose tissue and insulin release from pancreatic cells also play important roles in feeding (Cummings and Overduin, 2007; Huda et al., 2006; Moran, 2009; Smith, 2000). Neurons within the arcuate (ARC) and lateral (LH) hypothalamic nuclei sense these signals and can initiate or cease food seeking and consummatory behaviors in response to changes in the levels of these hormones (Morton et al., 2006; Smith, 2000; Woods and D'Alessio, 2008). Thus, humans and animals sense hunger and fullness through a change in metabolic state, hormonal balance, and the cascade of neural events set off by such changes (Figure 1.1).

However, feeding and related behaviors, such as food seeking and choice are complex and far from completely understood. Homeostatic signals are not the only driving force behind food consumption, as other neural sites are also critical in appetite regulation and food choice behaviors (Grill, 2006; Lutter and Nestler, 2009).
Figure 1.1. Cascade of signals and other responses from gastrointestinal tract following food intake and brain centers recruited during feeding. This highly simplified version of the gastrointestinal responses which are sensed by organs such as the pancreas, liver, adipose tissue, muscle, and the CNS. During food intake, a variety of mechanical and hormonal responses occur which are sensed by vagal and taste afferents. These afferents, along with circulating levels of hormones that may cross the blood-brain-barrier, lead to information processing by the brain through a complex network of homeostatic, reward, and cognitive neural sites to mediate food choice, initiation or cessation signals, and other information such as the environmental context, and then elicits the appropriate motor responses towards or away from the food. Amy: amygdala; AP: area postrema; ARC: arcuate nucleus of the hypothalamus; CCK: cholecystokinin; DMX: dorsal motor nucleus of the vagus nerve; GIP: gastric inhibitory peptide; GLP-1: glucagon-like peptide-1; Hipp: hippocampus; LH: lateral nucleus of the hypothalamus; NAcc: nucleus accumbens; PVN: paraventricular nucleus of the hypothalamus. PYY: peptide YY.

Along with the hypothalamic system, the central reward pathway, also known as the mesolimbic dopamine system, becomes engaged during food seeking and consumption.
Often, this type of consumption is driven less by hunger or satiety signals, and more by palatability, or a strong positive hedonic valuation of the food (Berridge and Robinson, 2003; Berridge et al., 2010; Egecioglu et al., 2011; Levine et al., 2003a; Saris, 2003). Palatable foods tend to be high in fat and sugar content, and while many of these foods are now highly processed and contain little or no nutritive value, they nevertheless have an extremely positive hedonic value to the consuming organism (Egecioglu et al., 2011; Levine et al., 2003; Small, 2009b). In fact, when the highly palatable food sucrose is consumed, dopamine release increases in the nucleus accumbens (NAcc), one of the key sites along the mesolimbic dopamine pathway receiving input from dopaminergic VTA neurons (Avena et al., 2006; Datla et al., 2002; Hajnal and Norgren, 2001; Rada et al., 2005). This occurs in a sweetness-dependent manner and regardless of post-ingestive feedback signals, as demonstrated using sham-feeding procedures which allow food to bypass gastrointestinal (GI) processing and thus minimize or avoid the GI responses normally seen after intake (Hajnal and Norgren, 2001; Hajnal and Norgren, 2002; Hajnal et al., 2004; Smith, 2004). Non-sweet, energy dense foods such as fats have also been tested in rats and shown to not only be preferred to water at concentrations as low as 0.78% corn oil, but like sucrose, a fatty solution such as corn oil was readily consumed up to concentrations of 50%, and sham feeding a 100% corn oil solution can produce DA increases despite minimal post-ingestive feedback signaling (Castonguay et al., 1984; Liang et al., 2006; Mindell et al., 1990).

Human studies have shown that the homeostatic and hedonic systems of the CNS are under complex regulation and are far from mutually exclusive (Berridge et al., 2010; Volkow et al., 2002; Wang et al., 2004a). In fasting human volunteers, swabs of subjects’ self-identified “favorite” foods increased hunger and craving along with producing significant increases in areas of the brain closely associated with taste perception (Wang et al., 2004a).
Of significant interest were that the “favorite foods”, self-chosen by a group of subjects ranging from normal to overweight (BMI range: 20-29), were all foods high in fat and carbohydrates, including pizza, cheeseburgers, cinnamon buns, fried chicken, lasagna, ice cream and chocolate items such as cakes and brownies (Wang et al., 2004a). Furthermore, studies of humans have shown that the mesolimbic dopamine system, as in rats, is indeed highly involved in feeding behaviors (Volkow et al., 2002; Wang et al., 2004b).

However, as repeated overconsumption of palatable foods occurs due to easy access, social acceptability, and strong environmental cues, food choices may be made due to factors other than the nutritional value of the food, and unhealthy choices may replace highly nutritive meals outside of times of caloric need. These factors, along with advertisements targeted toward pleasure engage the reward system, can make proper food choice difficult (Levine et al., 2003; Patel and Schlundt, 2001; Spangler et al., 2004; Volkow et al., 2008). Thus these highly palatable yet unhealthy foods can be, and often are, consumed in excess and are believed to be a major environmental factor in the rise of obesity rates (Fortuna, 2012; Kenny, 2011b; Volkow and Wise, 2005).

Information from the nucleus of the solitary tract (NTS) regarding taste perception (i.e. palatability) projects to other taste related nuclei and the central reward pathway (Morton et al., 2006; Norgren, 1976). These nuclei include the VTA, NAcc, thalamic nuclei, and cortical regions, which perceive and evaluate the hedonic value of the food (Morton et al., 2006; Volkow et al., 2011). With repeated exposure to highly palatable foods, the reward system is engaged in a manner similar to that elicited by drugs of abuse, such that it can create long-lasting changes in the individuals’ future motivation to procure and consume such foods (Gearhardt et al., 2011; Kenny, 2011a; Volkow et al., 2011). This can lead to an increased susceptibility for foods that were previously chosen and consumed due to their
hedonic nature, and eventually to overconsumption through an altered reward response. Studies have shown that sucrose given to rats regardless of caloric need can lead to adaptations in the mesolimbic dopamine system, altering the availability of dopamine receptors, and causing upregulation of both dopamine release and the dopamine transporter (DAT) (Avena et al., 2006; Avena et al., 2008; Bello et al., 2002; Bello et al., 2003; Colantuoni et al., 2001). Furthermore, sucrose produces neurochemical changes in the opioid system, which interacts heavily with the dopamine system along the central reward pathway (Colantuoni et al., 2001; Levine et al., 2003a). Not surprisingly, animal models of over exposure to palatable foods show that these neuroadaptational changes can produce serious alterations in behavioral responses, such as increased motivation to work for sucrose or other palatable foods, and binge-like behaviors (Avena et al., 2008; Avena et al., 2009; Corwin et al., 2011; Levine et al., 2003a).

Thus researchers for a number of years have speculated at the possibility of “food addiction,” as studies of palatable foods and drugs of abuse have often seen similar adaptations in behavior and the brain, and theorized as to the link between these complex aspects of feeding and the current obesity epidemic. To this end, a number of hormones such as ghrelin, leptin and insulin, previously associated as “homeostatic signals” are also being studied to determine their effects and interactions with the reward system (for review, see (Egecioglu et al., 2011)). Recently, increased attention has also been given to the incretin glucagon-like peptide-1 (GLP-1), as perhaps it serves as more than just a peripheral satiety hormone and incretin (Baggio and Drucker, 2007; Hayes et al., 2010).
1.2. Central and Peripheral Properties of GLP-1 in Feeding

GLP-1 was first identified for its importance as an incretin, that is, a hormone stimulating insulin secretions from pancreatic beta cells following stimulation from oral glucose, but not intravenous glucose (Doyle and Egan, 2007; Phillips and Prins, 2012; Wang et al., 1995). GLP-1 is produced by posttranslational processing of the proglucagon gene (encoded in the long arm of chromosome 2), which is expressed in intestinal L cells, pancreatic alpha cells, and neurons in the caudal brainstem (Baggio and Drucker, 2007; Drucker, 1998; Phillips and Prins, 2012). Processing is tissue-specific, with proconvertase (PC)1/3 in L cells and the brain, and PC2 in alpha cells, cleaving proglucagon to produce the active versions GLP-1(7-37) and GLP-1 (7-36) amide (Baggio and Drucker, 2007; Holst, 2007).

GLP-1 release is triggered by consumption of carbohydrates, fats, or a mixed meal; the postprandial response is biphasic with the initial release within 10-15 mins and a delayed peak response at 60 mins (Drucker, 1998; Reimann and Gribble, 2002; Vahl et al., 2010). Intestinal secretion of GLP-1 then triggers augmentation of glucose-dependent insulin release, decreases in glucagon, delayed gastric emptying, inhibition of meal-stimulated gastric acid secretion, increased peripheral glucose disposal to reduce blood glucose levels, and promotes satiety (Ahren, 2011; Chelikani et al., 2005; Drucker, 1998; Gutzwiller et al., 1999; Gutzwiller et al., 2004; Naslund et al., 1998; Phillips and Prins, 2012). Thus peripheral GLP-1 appears to not only regulate postprandial glycemia, but also have a significant effect on the enteric system to promote satiety (Blundell and Naslund, 1999; Flint et al., 2001; Phillips and Prins, 2012). This occurs despite the very short half-life of the bioactive form of GLP-1 (7-36) amide (< 5 mins), due to enzymatic degradation by dipeptidyl-peptidase 4 (DPP-IV), converting GLP-1 to its metabolite GLP-1 (9-36) amide (Baggio and Drucker,
2007; Phillips and Prins, 2012). Despite this short half-life, intravenous GLP-1 has been shown to reduce food intake (Chelikani et al., 2005; Flint et al., 2001). However, most studies of the peripheral GLP-1 system have instead utilized the longer-lasting synthetic GLP-1 mimetic, Exendin-4 (Ex-4), which produces robust changes in food intake when administered peripherally (Bojanowska and Nowak, 2007; Kanoski et al., 2011; Mack et al., 2006).

Of particular interest are the recent discoveries that GLP-1 may interact with the peripheral taste system. GLP-1 has shown to be colocalized with markers for taste receptors in both the gut and on the tongue. On the tongue, GLP-1 was coexpressed with markers for taste transduction elements such as α-gustducin and T1R3, which forms the sweet taste receptor by heterodimerizing with T1R2 (Shin et al., 2008). In the gut, L-cells expressing α-gustducin and T1R3 are crucial for normal GLP-1 secretion, and GLP-1 receptor knockout (KO) mice exhibit reduced sensitivity to sweet tastes (Höfer et al., 1996; Jang et al., 2007; Kokrashvili et al., 2009a; Kokrashvili et al., 2009b; Martin et al., 2009; Rozengurt et al., 2006; Shin et al., 2008). This indicates that the peripheral taste system is inherently linked to the GLP-1 system, and there appears to be serious implications for changes in feeding behaviors if either system is altered drastically (Martin et al., 2009; Shin et al., 2008).

While these and other effects occur in the periphery, they are likely under the control of central mechanisms as well (Hayes et al., 2008; Hayes et al., 2009; Kanoski et al., 2011). The early release of GLP-1 is thought to arise from signals in the CNS, and central GLP-1 appears to be highly involved in the control of food intake, perhaps above and beyond actions in the periphery (Hayes et al., 2008; Hayes et al., 2009; Moran, 2009; Murphy and Bloom, 2004; Tang-Christensen et al., 1996; Turton et al., 1996; Vahl et al., 2010). It is widely accepted that GLP-1 administered directly in the ventricular system of the brain can potently
reduce normal food intake in hungry rats in a dose-dependent manner (Asarian et al., 1998; Tang-Christensen et al., 1996; Turton et al., 1996). Furthermore, these effects are GLP-1 receptor dependent, as they can be blocked or reversed by GLP-1 receptor antagonists such as Exendin-3 (9-39) amide (Ex-9) (Hayes et al., 2009; Turton et al., 1996; Williams et al., 2009).

As it became clear that central GLP-1 was involved in feeding, other studies naturally investigated the effects of GLP-1 on specific nuclei. Attention turned to what nuclei are activated by centrally applied GLP-1, which included the area postrema (AP), and the arcuate (ARC) and paraventricular (PVN) nuclei of the hypothalamus (Larsen et al., 1997b; Tang-Christensen et al., 2001; van Dijk et al., 1996). Interestingly, GLP-1 also activated taste nuclei including the nucleus of the solitary tract (NTS) and the lateral parabrachial nucleus, indicating that the central taste system may be interacting with GLP-1 as well (van Dijk et al., 1996). In fact, within the brain, GLP-1 is produced primarily within the NTS, and to some extent within the olfactory bulb and parts of the reticular nucleus (Jin et al., 1988; Larsen et al., 1997a; Merchenthaler et al., 1999; Tang-Christensen et al., 2001). Furthermore, GLP-1 receptors are even more widespread throughout the brain. Receptors have been located within the NTS and area postrema, the arcuate nucleus, and other hypothalamic nuclei, among other sites (Alvarez et al., 1996; Alvarez et al., 2005; Goke et al., 1995; Uttenthal et al., 1992). GLP-1 receptors have not only been located among circumventricular organs such as the area postrema, but the ability of GLP-1 to cross the blood-brain-barrier (BBB) via simple diffusion has also been demonstrated (Kastin, 2001).

Given the location of GLP-1 receptors, it is not surprising that studies have shown intracerebroventricular GLP-1 is highly effective at suppressing food intake (Tang-Christensen et al., 1996; Turton et al., 1996). A number of studies have shown that GLP-1
receptor activation, by exogenous GLP-1 or the synthetic analog Ex-4, reduces food intake during homeostatic need, albeit the responses to Ex-4 and GLP-1 do differ in duration and intensity (Asarian et al., 1998; Barrera et al., 2009; Donahey et al., 1998; Tang-Christensen et al., 1996; Turton et al., 1996). While the effects of GLP-1 on homeostatic feeding have been extensively investigated, less is known of how GLP-1 may influence non-homeostatic feeding, such as palatability-driven intake. However, two early studies indicate that GLP-1 can reduce the intake of a palatable food, sucrose, regardless of post-ingestive feedback signals (Asarian et al., 1998; Chelikani et al., 2005). The sites and mechanism of action surrounding GLP-1’s role in hedonic and food intake, however, had yet to be fully explored.

**1.3. GLP-1, obesity, and Roux-en-Y gastric bypass surgery: A complex series of changes**

The currently available data on how the GLP-1 system is altered in diet-induced obesity, and how GLP-1 receptor activation may influence the feeding behaviors of obese individuals, is limited to the peripheral GLP-1 system and is still contradictory. There remains a debate in the literature as to the extent to which GLP-1 plasma concentrations are altered in obesity. Some reports have observed attenuated GLP-1 plasma levels in obese animals and humans, which in some studies has been shown to return back to normal levels following weight loss (Adam and Westerterp-Plantenga, 2005; Anini and Brubaker, 2003; Holdstock et al., 2008; Ranganath et al., 1996; Verdich et al., 2001). Other data from obese individuals indicates no change or even an increase in either fasting or postprandial GLP-1 concentrations in the blood (Bojanowska, 2005; Fukase et al., 1993; Iritani et al., 1999). However, when administered exogenously to obese individuals, GLP-1 still lowers subjective hunger ratings and the amount of food consumed to levels comparable to lean controls,
indicating that GLP-1 may retain some efficiency even in cases of obesity (Flint et al., 2001; Naslund et al., 1998; Naslund et al., 1999).

Regardless of this debate, several reports do clearly demonstrate that Roux-en-Y gastric bypass surgery (RYGB) improves GLP-1 levels in obese patients and rats alike, concurrent with changes in appetite and taste preferences, but prior to actual weight loss (Hajnal et al., 2010a; Ionut and Bergman, 2011; le Roux et al., 2006; Morinigo et al., 2006; Pournaras et al., 2010; Shin and Berthoud, 2011; Shin et al., 2011; Tam et al., 2011). It can be theorized that changes in gut-brain signaling, such as GLP-1, may strongly contribute to the food intake, food reward, and taste changes following RYGB (Hajnal et al., 2010b; Mathes and Spector, 2012; Shin et al., 2011; Tichansky et al., 2011). Indeed, studies of GLP-1, alone or in combination with other hormones that are altered during obesity and RYGB, may lead to a better understanding of the underlying mechanisms behind the success of the surgical procedure. In fact, such an understanding could lead to non-surgical alternative treatments targeting the problem of obesity and its associated metabolic consequences. Interestingly, one such metabolic issue, type 2 diabetes mellitus (DM), has been shown to respond to GLP-1 therapy with efficacious results. Along with DM, GLP-1 may prove to have other, highly important clinical implications.

1.4. CURRENT CLINICAL RELEVANCE OF GLP-1

Among the two major incretins, GLP-1 and gastric inhibitory peptide (GIP), only GLP-1 retains it’s function as an incretin in patients with type 2 diabetes (Drucker, 1998; Phillips and Prins, 2012). While both potentiate glucose-induced release of insulin, only GLP-1 retains this function in patients with type 2 DM, despite decreases in GLP-1 secretion in these patients (Drucker, 1998; Nauck et al., 2004; Nauck, 2011; Phillips and Prins, 2012).
As GLP-1 appeared to play a crucial role in the control of postprandial blood sugar, and the receptor appeared to remain sensitive to GLP-1 during type 2 DM, previous studies initially investigated how GLP-1 itself may be an alternative treatment for type 2 DM patients. These studies found that GLP-1 treatments could mediate glycemia in healthy patients, normalize postprandial glucose levels in patients with type 2 DM, and produce some weight loss due to inhibited gastric emptying, with minor negative side effects such as nausea (Edwards et al., 1998; Nauck et al., 1996; Nauck et al., 1998; Ritzel et al., 1995; Todd et al., 1998; Zander et al., 2002). However, due to the rapid degradation of GLP-1 by DPP-IV, supraphysiological levels of GLP-1 were required and thus longer-lasting GLP-1 receptor (GLP-1R) agonists have been identified and are now used with equivalent success (Drucker, 1998; Parkes et al., 2001; Phillips and Prins, 2012).

In 2005, the US Food and Drug Administration (FDA) approved Exenatide, marketed as Byetta® by Amylin Pharmaceuticals, for the treatment of type 2 DM. This synthetic form of the GLP-1R agonist Ex-4 has a longer half-life than exogenously applied GLP-1 (2.4 hrs compared to less than 5 min), and has shown to be successful in treating hypoglycemia (Bond, 2006; Lovshin and Drucker, 2009; Phillips and Prins, 2012). However, Exenatide had to be given as an injection several times a day (Bond, 2006). Soon thereafter, Exenatide was modified to be a once weekly injection (EQW), and showed the same success at controlling glycemia in type 2 DM patient populations (Balena et al., 2012; Pencek et al., 2012). This version of the GLP-1 agonist was also recently approved by the US FDA with the trademark name Bydureon® through Amylin Pharmaceuticals in January 2012 (US FDA and RESEARCH, 2012).

Another long-acting synthetic GLP-1 agonist, liraglutide was also approved by the FDA in 2010 for the treatment of type 2 DM (Phillips and Prins, 2012). Marketed by Novo
Nordisk as Victoza®, it is considered an effective alternative to Byetta®, as patients experienced slightly different responses and side effects to the two drugs (Buse et al., 2009; Buse et al., 2010). Another initial advantage of liraglutide was that it was a once daily injection, rather than the multiple injections of Exenatide, although Exenatide QW has now surpassed that beneficial aspect of the drug. Despite minor differences, these drugs have been shown successful through clinical trials and current usage by patient populations to regulate fasting and postprandial blood sugar levels (Phillips and Prins, 2012). As animal models have used these drugs to study feeding behaviors in healthy and obese rodent models, recent attention has also been given to how these drugs may influence body weight of human subjects (Bradley et al., 2012; Hayes et al., 2010; Hayes et al., 2011). Further research is needed to fully understand how GLP-1 influences homeostatic hunger, hedonic eating, and food reward. However, current models of obesity and RYGB being utilized should lead to a better understanding of how to successfully use GLP-1 agonists such as these for the treatment of food craving, overeating, and obesity.

1.5. Dopamine and the Cycle of Overeating: Theories for GLP-1

To fully understand the therapeutic potential for GLP-1 in overeating and body weight control, the precise mechanism of action and interactions of GLP-1 within the brain must be carefully understood. As previously described, GLP-1 has been shown to modulate food intake based on both nutritional and palatability attributes, and to have a strong relationship with the peripheral taste systems, both oral and GI. Furthermore, considering the pattern of changes with GLP-1 following obesity and RYGB surgery, and the success of GLP-1 agonists in the treatment of type 2 DM with minimal side effects, the potential impact for GLP-1 as a potential therapy for hedonic eating and food reward must be explored. To
fully appreciate this potential, the mechanism of action for GLP-1 within the central reward system, which is believed to be highly crucial to the cycle of overeating, will be explored in the experiments within the following chapters.

As previously described, the central reward system becomes engaged during feeding through signals arising from the GLP-1-producing NTS, which initially processes taste information and then projects that information to a number of nuclei for further valuation and processing (Morton et al., 2006). Along with hypothalamic and cortical nuclei, this information is also received by the VTA and areas relevant to secondary attributes of food such as smell, sight, and general cognitive information (Morton et al., 2006). The VTA then projects to the NAcc, striatum, and other areas that may encode relevant secondary information such as context, such as the amygdala and cortex (Berridge and Kringelbach, 2008; Swanson, 1982). Information is also bidirectionally sent from the NAcc to the amygdala, hippocampus, and cortical areas such as the prefrontal and orbitofrontal cortices. This highly processed information eventually feeds back to the NTS, while the VTA receives extensive input and feedback from the hypothalamus, NAcc, amygdala, hippocampus, and similar cortical regions (Kelley and Berridge, 2002; Morton et al., 2006; Wise, 2006). This complex reward circuit, in which dopamine (DA) is a key neurotransmitter, has received extensive attention for its role in hedonic feeding behavior and food reward, and has been shown to react to exposure to palatable foods in a similar manner as drugs of abuse (Kelley and Berridge, 2002; Morton et al., 2006; Volkow et al., 2008; Volkow et al., 2011). In fact, in response to palatable food intake, the information encoded by the VTA leads to conditioned responses to rewards and associated cues, leading to increased salience for the food, termed “wanting” by Berridge and colleagues (Berridge, 2009; Kelley and Berridge, 2002; Volkow et al., 2008; Volkow et al., 2011; Wang et al., 2001; Wise, 2006). Thus with repeated
exposure to palatable foods, the response of DA within the central reward pathway is altered to predict reward and promote engagement in behaviors to procure and consume the food (Bello et al., 2002; Bello et al., 2003; Bernal et al., 2009; Bernal et al., 2008; Berridge and Robinson, 1998; Berridge and Kringelbach, 2008; Gearhardt et al., 2011; Kelley and Berridge, 2002).

Obesity has been shown to significantly alter the neurochemical and behavioral responses to highly palatable foods. Indeed, even obese children show hyperresponses to pictures of palatable foods with hyperactivation in the prefrontal cortex, prelimbic and limbic areas, such as the NAcc, that encode reward and motivation (Bruce et al., 2010). A number of other studies in adults have shown that obese individuals, when presented with either a taste or image of a palatable food, show hypersensitivity in areas of feeding such as the hypothalamus, as well as areas related to reward and motivation such as the OFC, NAcc, dorsal striatum, amygdala, hippocampus, and insular and anterior cingular cortices (Killgore and Yurgelun-Todd, 2005; Rothemund et al., 2007; Volkow et al., 2011; Wang et al., 2004a; Wang et al., 2004b).

Given the changes observed in both the GLP-1 system and the dopamine system during obesity, it seems highly likely that the GLP-1 may play an important role in modulating intake of palatable foods, and that in doing so it may crucially interact with the central reward system, and particularly dopamine. Furthermore, in cases of obesity both the GLP-1 and dopamine system may be altered. Despite these important implications, changes to the GLP-1 system in obesity is still under debate, and how obese individuals may respond to GLP-1 during tests of hedonic intake and food reward have yet to be investigated. Thus in Chapters 2 and 3, we investigate how dietary induced obesity (DIO) may influence both the responsivity of the DA and GLP-1 systems using similar cohorts and testing procedures,
while manipulating DA and GLP-1 using peripherally administered, receptor-specific drugs. In Chapter 4, the role of GLP-1 within the brain is extensively investigated to determine areas of action. Once the involvement of the VTA was established, we further investigated the relationship between GLP-1 and the VTA dopaminergic system using immunohistochemistry and electrophysiology. Finally, in Chapter 5 we utilized an animal model of RYGB in DIO rats to investigate how the surgical procedure may influence responding, how manipulations of the GLP-1 system may change responding in this cohort, and how RYGB may influence levels of GLP-1 receptors within the brain.
Chapter 2.

**Obesogenic diets may differentially alter dopamine control of sucrose and fructose intake in rats**

* This chapter includes results from the manuscript: Pritchett CE, Hajnal A. (2011). Obesogenic diets may differentially alter dopamine control of sucrose and fructose in rats. *Physiology and Behavior, 104*(1), 111-116. See Appendix for published manuscript.

2.1. Introduction

Decades of research by Hoebel and his trainees have provided essential information on the role of the brain's dopaminergic system in the regulation of feeding, thus developing the concept of "food reward" (Avena et al., 2006; Hernandez and Hoebel, 1988a; Hernandez and Hoebel, 1988b; Rada et al., 2005). Remarkably, Bart Hoebel's early experiments established midbrain dopamine as a key factor in chronic overeating and resultant obesity (Ahlskog, 1976; Ahlskog et al., 1984; Hernandez and Hoebel, 1982; Hoebel et al., 1981), long before direct evidence was made available from imaging studies (Stice et al., 2008; Volkow et al., 2011).

The notion that food exerts control over eating, and in turn, that sustained or intermittent access to highly palatable meals (i.e. those high in sugars and fats) could cause lasting changes within feeding regulatory systems has long been central to Hoebel's theory on the development of binge-type behaviors. Early in his career, he also applied elements of this reasoning to obesity. In a 1977 review, Hoebel remarked that there may be "different kinds of obesities that require different treatments" (Hoebel, 1977). Since then, a plethora of research on obesity has indeed identified various genetic, metabolic, and environmental factors that may explain the variation in the development, consequences, and treatment of obesity (Bouchard, 1991; Small, 2009a; Vogele, 2005; Weinsier et al., 1998). However, our understanding of the specific contributions of macronutrients to altered food reward...
functions is far from complete. The present paper summarizes data from a study that was inspired by Bart's research and intended to reduce this gap in our knowledge. Within the multifaceted etiology of obesity, diet remains a key factor in obesity development. Obesogenic diets are diets high in caloric value, often palatable foods that lead to obesity after extended exposure (Archer and Mercer, 2007). However, the macronutrient composition of obesogenic diets can differ and this variation could impact neural systems altered in obesity, such as dopamine. Indeed, feeding rats an obesogenic diet has been shown to reduce dopamine levels in the accumbens, as well as shift the reactivity of the mesocorticolimbic system such that a more palatable diet is required to achieve similar food-induced increases in extracellular dopamine as seen in chow-fed controls (Geiger et al., 2008). One potential mechanism is an adaptive down-regulation due to augmented and chronic stimulation by palatable foods (Volkow et al., 2011). In fact, studies from our laboratory have shown that even orosensory stimulation by either sucrose or fat is sufficient to increase dopamine release in the rat nucleus accumbens (Hajnal et al., 2004; Liang et al., 2006). Of particular relevance, fat and sugars appear to affect reward systems differently, as it is inferred from the greater potency of sugars to produce addictive-like behaviors (Avena et al., 2009). Other recent investigations have shown differential effects on the neuroendocrine system and later susceptibility to weight gain based on the ratio of fat and carbohydrates in obesogenic diets (Shahkhalili et al., 2011; van den Heuvel et al., 2011). In addition, increased attention has been devoted to potential particularities in the regulatory responses to a high fructose corn syrup diet and the purported consequences of the apparent ease with which it can cause obesity and derangement of food regulation. Specifically, recent studies by Avena and Hoebel demonstrated that rats with access to high-fructose corn syrup (HFCS) for 12 h every day for 8 weeks gained significantly more body weight than animals given equal
access to 10% sucrose, even though they consumed the same number of total calories, but fewer calories from HFCS than sucrose (Bocarsly et al., 2010). The rising incidence of obesity and potential for the discovery of novel treatments demands investigation of how the intake of common high-energy and palatable foods, such as sucrose and fructose, is controlled under dietary obesity conditions. Therefore the current study investigated dopamine regulation of sucrose and fructose intake in rats that became obese as a result of extended maintenance on two standard high-energy diets widely used to produce dietary obesity in rats, varying in fat and carbohydrate content. Specifically, we evaluated the effects of the two major classes of dopamine receptors using peripheral (intraperitoneal; i.p.) administration of the dopamine D1 receptor (D1R) antagonist SCH23390 or the dopamine D2 receptor (D2R) antagonist raclopride in lean and dietary obese rats on a short (2-h) intake of sucrose or fructose. These common carbohydrates are prevalent within human diets, are readily consumed by rats and have positive reinforcing properties (Ackroff et al., 2001; Reedy and Krebs-Smith, 2010; Sclafani, 1987; Sclafani et al., 1998). Sucrose intake has previously been shown to stimulate dopamine release within the nucleus accumbens (Avena et al., 2006; Hajnal and Norgren, 2002; Hajnal et al., 2004) and peripheral administration of both SCH23390 and raclopride reduces sucrose sham-feeding (Weatherford et al., 1990). Although there is heightened interest by the science community, as well as the public media, similar effects of dopamine antagonists on fructose intake have only been investigated in the context of acquisition and expression of conditioned preferences, and these studies were also limited to lean rats (Baker et al., 2003; Bernal et al., 2009; Bernal et al., 2008). Despite the potential implications, effects of dopamine receptor antagonists on carbohydrate intake in various obesity models and in the absence of homeostatic drive (i.e. following periods of
food restriction) have not been investigated. Therefore, rats in the current study were kept sated to avoid confounding effects from hunger and energy deficit.

2.2. Methods

2.2.1. Animals and diets

Twenty-eight adult male Sprague–Dawley rats (Charles River, Wilmington, MA) weighing approximately 250 g at the onset of the study were housed in individual cages in a temperature-controlled vivarium and maintained on a 12:12 light–dark cycle, with lights on at 0700. Animals were given ad libitum access to one of the following three diets: standard laboratory chow (Teklad #2018, 3.4 kcal/g, 18 kcal% fat, 58 kcal% carbohydrates, 24 kcal% protein; Teklad Diets, Somerville, NJ) or one of two high-energy diets (Research Diets, New Brunswick, NJ), one diet in which the primary energy source was fat (high fat-high energy, HFHE diet; Research Diets #D12492: 5.24 kcal/g, 60 kcal% fat, 20 kcal% carbohydrates, 20 kcal% protein) or a high-energy diet consisting of both fat and carbohydrates (fat–sugar combination high energy, FCHE diet; Research Diets #D12266B; 4.41 kcal/g, 32 kcal% fat, 51 kcal% carbohydrates, 17 kcal% protein). At the beginning of the study, groups were weight matched to form statistically equal cohorts based on body weight and were then maintained on their respective diets for 24 weeks prior to and throughout the behavioral experiments. Starting at 18 weeks and throughout the experiment, body weight and food intake were measured daily. Animals were tested in a sated state with no periods of food restriction throughout the experiment.

2.2.2. Body composition

In addition to significant increase in body weight, to demonstrate the presence of obesity (defined as a significantly higher percentage of body fat relative to chow-fed controls) $^1$H-
NMR body composition analysis (Bruker LF90 proton-NMR Minispec; Brucker Optics, Woodlands, TX) was performed after 12 weeks of maintenance on the diets.

2.2.3. Dopamine antagonists, test solutions, and testing procedure

The dopamineD1R antagonist SCH23390 (HFHE: n=6; FCHE: n=5; Chow: n=4) and the dopamine D2 receptor antagonist raclopride (HFHE: n=5; FCHE: n=6; Chow: n=4) were used. SCH23390 and raclopride (Tocris Biosciences, Ellisville, MO) were dissolved in sterile saline and administered intraperitoneally 10 min prior to 2-h access to 0.3 M sucrose or 0.4M fructose. These concentrations were chosen as they are highly palatable to rats and have therefore been commonly used in previous studies (Avena et al., 2006; Baker et al., 2003; Hajnal et al., 2004; Smith, 2004). Sucrose and fructose (Fisher-Scientific, Fair Lawn, NJ) were dissolved in filtered tap water no more than 24 h prior to testing.

Animals were trained to drink test solutions during daily sessions where 2-h access (beginning at 1000 h) to sucrose or fructose was provided for 8 days prior to testing to achieve stable baseline intakes, i.e. familiarity with the orosensory and post-ingestive effects. Training and testing took place in the animals' home colony room, with 100 ml plastic bottles temporarily attached to the front of the home cage so that spouts extended into the cage. Administration of vehicle (saline) or dopamine antagonists began after 24 weeks of maintenance on the diets, at which point both obesogenic diet groups (HFHE and FCHE) had significantly higher body weights than chow controls (Figure 2.1). A minimum of 48 h was given between injection days to allow drugs to completely metabolize. No changes to body weight or 24-hour food intake occurred following treatment with the dopamine antagonists.

2.2.4. Statistical analysis

Body weight and 1H-NMR data were analyzed using a one-way independent samples analysis of variance (ANOVA) with diet as the independent variable. Intake was measured as
ml consumed and presented as mean ± SEM. Baseline intake (following vehicle, i.e. saline injection) was tested for differences between the diet groups in a three way ANOVA with diet, drug, and carbohydrate as the independent variables. There were no significant effects of Diet (F(2,48)=0.3533, p=0.704), Drug (F(1,48)= 0.1482, p=0.701), nor were there significant interaction effects (Diet x Drug: F(2,48)=0.4144, p=0.66; Diet x Carbohydrate: F(2,48)=0.2759, p=0.76; Drug x Carbohydrate: F(1,48)=0.0062, p=0.73; Diet x Drug x Carbohydrate: F(2,48)=0.3108, p=0.73). However, a significant effect of Carbohydrate (F(1,48)=8.8974, p<0.01) was observed (Table 2.1).

Table 2.1. Intake of sucrose and fructose in 2-h tests. Absolute intake values (in ml) of sucrose and fructose intake by diet groups following vehicle (0 nmol/kg) injections. No differences were observed in baseline intake between diet or drug groups. Baseline sucrose intake was significantly greater than baseline fructose intake (p<0.01). Therefore, for all further analyses of drug effects, changes were normalized and expressed as a percent change relative to vehicle baseline intakes.

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>Drug</th>
<th>Diet Group</th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>SCH23390</td>
<td>HFHE</td>
<td>20.33 ± 3.54</td>
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<tr>
<td></td>
<td></td>
<td>FCHE</td>
<td>21.20 ± 2.85</td>
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<td></td>
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<td>Chow</td>
<td>21.00 ± 2.65</td>
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<tr>
<td></td>
<td>Raclopride</td>
<td>HFHE</td>
<td>21.00 ± 2.35</td>
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<td></td>
<td></td>
<td>FCHE</td>
<td>21.00 ± 4.14</td>
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<tr>
<td></td>
<td></td>
<td>Chow</td>
<td>22.25 ± 2.02</td>
</tr>
<tr>
<td>Fructose</td>
<td>SCH23390</td>
<td>HFHE</td>
<td>15.67 ± 2.14</td>
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<tr>
<td></td>
<td></td>
<td>FCHE</td>
<td>17.2 ± 2.85</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chow</td>
<td>12.5 ± 2.02</td>
</tr>
<tr>
<td></td>
<td>Raclopride</td>
<td>HFHE</td>
<td>12.8 ± 2.83</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FCHE</td>
<td>17.67 ± 4.38</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chow</td>
<td>17.50 ± 2.36</td>
</tr>
</tbody>
</table>

Therefore, for all subsequent analyses intake was expressed as a percent reduction from baseline (intake following dose X [ml] / intake following 0 μg/kg [ml]) and analyzed using
repeated measures ANOVA with Diet (HFHE, FCHE, or Chow) and Drug (raclopride or SCH23390) as independent variables and dose (0, 50, 200, 400 or 600 nmol/kg SCH23390 or raclopride) as the repeated measure. The inhibitory dose (ID50) required to reduce intake to 50% of baseline (0 nmol/kg) was calculated as previously described (Hajnal et al., 2007b).

Differences in ID50 were compared as a function of Diet and Drug using two-way ANOVA. All analyses were conducted using Statistica (v6.0, StatSoft® Inc., Tulsa, OK) and significant findings were further analyzed using Fischer's least significant difference (LSD) post-hoc tests. Differences were considered statistically significant if p<0.05.

2.3. Results
2.3.1. Effects of diet on body weight and adiposity

After 12 weeks on the obesogenic diets, the groups differed in body weight (F(2,27)=27.25, p<0.001), percent fat mass (F(2,27)=14.96, p<0.001), and percent lean mass (F(2,27)=15.77, p<0.001). The post hoc tests showed that Chow rats weighed significantly less than both HFHE (p<0.001) and FCHE (p<0.001) rats. Comparison of body composition showed that HFHE and FCHE rats had a greater percentage of fat mass compared to Chow (p<0.05). At 18 weeks, at the beginning of testing (24 weeks), and throughout the testing period, there remained a significant effect of diet on body weight (Figure 2.1 week 18: F(2,27)=13.05, p<0.001; week 24: F(2,27)=16.96, p<0.001; week 26: F(2,27)=13.99, p<0.001; week 28: F(2,27)=13.05, p<0.001). Post hoc analysis revealed that HFHE and FCHE rats had significantly higher body weights than Chow controls (Figure 2.1; p<0.001, all time points). There were no statistical differences in body weight between the two obese groups at any time point.
Figure 2.1. Body weight during the period prior to and throughout pharmacological testing (gray bar). Rats maintained on HFHE or FCHE diets had significantly higher body weight than Chow rats at all times points. There was no difference in body weight between HFHE and FCHE groups at any time point. * p<0.001 compared to Chow.

2.3.2. Effects of dopamine D1R and D2R antagonism on sucrose intake

Sucrose intake was reduced by SCH23390 in all groups (Figure 2.2A). Raclopride reduced sucrose intake in HFHE rats, but was much less effective in Chow and FCHE rats (Figure 2.2B). The repeated measures ANOVA showed an overall effect of Drug (F(1,24)=2.9799, p<0.05). Whereas the overall effect of Diet was not significant (F(1,24)=2.5787, p=0.09), post hoc comparisons did show significant differences of raclopride treatment between HFHE and Chow groups (p<0.05) and between HFHE and FCHE groups (p<0.05). Post hoc analysis revealed that SCH23390 was significantly more effective at reducing sucrose intake overall compared to raclopride (p<0.01). SCH23390 suppressed sucrose intake in HFHE rats at all doses tested and suppressed intake in FCHE and Chow rats at 200 nmol and higher
doses (Figure 2.2A). Sucrose intake was suppressed in HFHE rats by all doses of raclopride, but only the highest dose reduced sucrose intake significantly in FCHE rats, while none of the doses suppressed sucrose intake by Chow rats (Figure 2.2B).

Analysis of the ID50 (Table 2.2) revealed no effect of Diet (F(2,24) =0.576, p=0.57) or Drug (F(1,24)=2.988, p=0.09), despite apparent differences in the ID50 for raclopride. This lack of an effect could be due to the substantial variance within groups.
Figure 2.2. Changes in sucrose intake following dopamine receptor antagonists. All data depicted as a reduction from baseline intake referring to vehicle injection (set at 1.0 or 100% on y-axis). A. Sucrose intake was significantly reduced by SCH23390 in all groups, with the most potent reduction occurring in HFHE rats. B. Raclopride reduced sucrose intake in HFHE rats at all doses tested and reduced intake by FCHE rats only at the highest tested dose. None of the doses significantly reduced sucrose intake in Chow rats. * p<0.05; ** p<0.01; *** p<0.001 compared to vehicle.

2.3.3. Effects of dopamine D1R and D2R antagonism on fructose intake

SCH23390 reduced fructose intake in all groups (Figure 2.3A). Raclopride, on the other hand, only reduced intake significantly in the FCHE group (Figure 2.3B). The repeated measures ANOVA revealed an overall effect of Drug (F(1,24)=5.7400, p<0.05), Dose (F(4,96)=33.9351, p<0.001) and a significant Dose by Drug interaction (F(4,96)=3.0296, P<0.05) but no effect of Diet (F(2,24)=1.5205, p=0.24). Again, however, post hoc analyses showed a significant difference of raclopride treatment between HFHE and FCHE groups (p<0.05). Post hoc analysis revealed that SCH23390 was overall more effective at suppressing fructose intake than raclopride (p<0.05), and did so in a dose-dependent manner.
(Figure 2.3). SCH23390 reduced intake in all diet groups at 400 and 600 nmol and reduced fructose intake as early as the 200 nmol dose in HFHE rats (Figure 2.3A). Raclopride effects on fructose intake, however, were limited to FCHE rats with post hoc analysis revealing significant reductions in fructose consumption in FCHE rats at 200 nmol and higher doses, with none of the raclopride doses suppressing fructose intake in HFHE or Chow rats (Figure 2.3B).
**Figure 2.3.** Changes in fructose intake following administration of dopamine receptor antagonists. All data depicted as a reduction from baseline intake referring to vehicle injection (set at 1.0 or 100% on y-axis). A. Fructose intake was significantly reduced by SCH23390. B. Raclopride reduced fructose intake in FCHE rats, but failed to reduce intake in HFHE or Chow rats at any dose. * p<0.05; ** p<0.01; *** p<0.001 compared to vehicle.

<table>
<thead>
<tr>
<th>Diet effect on ID&lt;sub&gt;50&lt;/sub&gt; (nmol/kg)</th>
<th>HFHE</th>
<th>FCHE</th>
<th>Chow</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SCH 23390 (D1R)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>384 ± 58</td>
<td>427 ± 160</td>
<td>325 ± 89</td>
</tr>
<tr>
<td>Fructose</td>
<td>175 ± 164</td>
<td>365 ± 54</td>
<td>307 ± 47</td>
</tr>
<tr>
<td><strong>Raclopride (D2R)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>678 ± 233</td>
<td>2114 ± 1345</td>
<td>1436 ± 715</td>
</tr>
<tr>
<td>Fructose</td>
<td>608 ± 115*</td>
<td>516 ± 99*</td>
<td>1677 ± 1007</td>
</tr>
</tbody>
</table>

**Table 2.2.** Effectiveness of dopamine receptor antagonists expressed as by ID50. The ID50 represents the dose at which intake would be reduce to 50% of baseline (vehicle). No differences were observed between groups for SCH23390. In contrast, raclopride was more effective at reducing fructose intake in HFHE and FCHE rats compared to lean Chow controls.
ANOVA on the ID50 (Table 2.2) revealed an effect of Drug (F(1,24)=4.548, p<0.05) but not Diet (F(2,24)=1.495, p=0.25). SCH23390 required lower doses overall than raclopride to reduce intake to half of baseline (p<0.05). In line with the analysis on actual doses, post hoc analysis of ID50 also revealed significantly increased sensitivity in both obese groups compared to Chow rats (p<0.05).

2.4. Discussion

The present study compared sensitivity to dopamine receptor blockade in reducing intake of two palatable carbohydrate solutions, sucrose or fructose, in two dietary obese animal models. We used two diets to mimic the chronic consumption of either a diet predominantly high in fat (HFHE), or a fat–sugar combination diet (FCHE), as occurs in the Western diet (Reedy and Krebs-Smith, 2010). As expected, both diets produced substantial weight gain and adiposity beginning at 12 weeks, with a continued increase in body weight throughout the experiment (Figure 1.1). The groups were then compared to age-matched chow-fed controls in their relative sensitivity to D1R and D2R subtype-specific blockade with SCH23390 or raclopride, respectively. We found that blockade of the D1Rs reduced both sucrose and fructose intake in all diet groups. Regardless of whether rats were consuming sucrose or fructose solutions, HFHE rats responded to slightly lower doses of SCH23390 compared to their obese FCHE or lean Chow counterparts (Figures 2.2A, 2.3A). This apparent increase in sensitivity to dopamine D1R antagonism by HFHE rats was also observed following D2R blockade during the sucrose test. Indeed, HFHE rats responded to all raclopride doses with reductions in sucrose intake, while FCHE rats only responded to the highest dose, and Chow rats showed no significant suppression of sucrose consumption following raclopride treatments (Figure 2.3B). Interestingly however, HFHE rats did not
reduce fructose intake following raclopride treatment. Instead, raclopride significantly suppressed fructose intake only in FCHE rats. An increased sensitivity to the dopamine receptor antagonists is indicative of reduced dopamine signaling, i.e. due to fewer receptors, a reduced competition from endogenous DA at the receptor sites, or a combination of both. In fact there is evidence that either mechanism may be applicable to our model. For example, exposure to high fat diets even before birth may result in decreased D2Rs (Naef et al., 2010). Furthermore, eating high fat food has shown to decrease natural or electrically evoked dopamine release, and attenuate dopamine turnover (Davis et al., 2008; Geiger et al., 2009; Rada et al., 2010). Whereas the underlying mechanism warrants further investigations, our data together with these and other previous observations support the notion that eating certain foods – potentially independent of obesity – may result in changes within the dopamine system reminiscent of neuroplasticity to drug of abuse (Koob and Volkow, 2009). In fact, recent research suggests that high fat diets increases sensitization to drugs acting on dopamine systems (Baladi and France, 2010; McGuire et al., 2011).

Previous investigations in lean rats have shown differential efficacy of D1R and D2R blockade to reduce carbohydrate intake using concentrations consistent with those used in the present study (Baker et al., 2003; Bernal et al., 2009; Bernal et al., 2008; Tyrka and Smith, 1993). These effects are believed to be partially mediated by areas of the brain involved in food reward, and D2Rs in these areas may be especially susceptible to changes caused by obesity (Bernal et al., 2009; Bernal et al., 2008; Johnson and Kenny, 2010; Volkow et al., 2008; Wang et al., 2001). The present study expanded upon the findings of dopamine receptor modulation of carbohydrate intake in lean rats and compliments those studies showing lasting plasticity in the reward system in obesity. Whereas complexity of the systems and factors that may influence such interplay (acute control of intake by a
chronically altered system) evidently increases individual variances and hence diminished interaction effects in the overall ANOVAs, the direct (post hoc) comparisons of dose–response effects did reveal differential sensitivity to isomolar doses of antagonists between the diet groups. Changes affecting the D2Rs specifically appeared to be dependent upon the content of carbohydrates also present in the high fat diets, indicating the macronutrient content of the diets could differentially alter the reward system.

The differential effects of sensitivity to raclopride in the sucrose test could be due to the presence of sucrose in the diets. Although both obesogenic diets contained some sucrose, the FCHE diet contained 23% more sucrose than the HFHE diet. Thus the lack of a response to raclopride when a sucrose solution was presented as the test stimulus to FCHE rats, but not HFHE rats, could have been due to the enhanced exposure to sucrose in the HFHE diet. However, neither obesogenic diet contained fructose, yet differences were observed in the responses of the obesogenic diet groups to raclopride in the fructose test as well. Furthermore, no sucrose was present in the Chow diet, yet responses by the Chow group to raclopride in the sucrose test were more akin to the responses made by FCHE than HFHE rats. This indicates that other factors than dietary adaptation may be underlying the differential responses to raclopride treatment as a function of diet and test carbohydrate.

Alternative explanations may include differential neural and hormonal post-ingestive effects exerted by fructose and sucrose. Whereas the exact mechanisms remain obscure, there is increasing evidence supporting this notion (Ackroff and Sclafani, 2011; Glendinning et al., 2010). In this context, the possibility that the two diets altered sucrose and fructose preferences differently as a result of their differential effects on oral and gastrointestinal signals upstream to the reward system cannot be excluded and warrants further investigation.

Obesity and palatable foods have independently been implied to alter dopamine
signaling (Avena et al., 2006; Bello et al., 2002; Hajnal et al., 2008; Johnson and Kenny, 2010), and therefore could also account for the differential responding observed in the present study. Indeed, our data support previous findings showing that dopamine D2R signaling is reduced in obesity (Bello et al., 2002; Johnson and Kenny, 2010). However, the novel finding of the present study was that the nature of this relationship may be dependent on the macronutrient content of the obesogenic diets rather than the presence of obesity. An additional major finding was the differences seen in the efficacy of D2R antagonists between test carbohydrates. We noted a trend in our data that fructose intake appeared to be more tightly controlled by D2Rs than sucrose intake, leading one to question how the intake of various carbohydrates may be differentially regulated, and if reward elicited by different carbohydrates may recruit varying mechanisms. Previous data has indicated that sucrose and fructose intake produce dissimilar physiological responses. Sucrose has been shown to produce conditioned effects based on both its taste and post-ingestive properties (Ackroff, 2008; Bonacchi et al., 2008; Sclafani et al., 1998) while fructose appears to exert behaviorally relevant stimulation exclusively by its taste and not by reinforcing post-ingestive effects (Ackroff et al., 2001; Sclafani and Ackroff, 1994). Therefore, responsiveness of reward circuitries to fructose may remain intact even when feedback elicited by sucrose becomes compromised due to impairments secondary to obesity (e.g. reduced insulin/leptin sensitivity). The opposite may also be true: a counter-regulatory response to curb sucrose intake may fail to check fructose intake. Future studies in humans are needed to investigate whether preferences for foods rich in fructose would actually increase with obesity, and if relative sucrose and fructose preferences are different in obese patients who are also diabetic.

While effects of sucrose on dopamine have been extensively investigated (Avena et
al., 2006; Bello et al., 2002; Hajnal et al., 2004; Tyrka and Smith, 1993), less is known of the interaction between fructose and the dopamine reward system, although early reports from the Hoebel lab indicate that fructose may produce its own unique physiological responses (Bocarsly et al., 2010). The present study adds a further piece of information to this complex puzzle suggesting that diets of different macronutrient content may differentially alter dopamine control of fructose intake. Further investigation is required to fully understand the underlying mechanisms by which dietary fat and sugar may influence gut–brain signaling and elicit changes within the brain.

2.5. Conclusions
This study demonstrates that obesogenic (high-energy) diets varying in fat and carbohydrate content, rather than obesity itself, may differentially increase sensitivity to D1 and D2 receptor antagonists in reducing carbohydrate intake. This finding is compatible with the general notion that dopamine signaling in dietary obesity is blunted, and suggests a novel relationship between diets and central dopamine effects. An additional major finding was that the diets differentially altered the potency of dopamine receptor antagonists in suppressing sucrose and fructose intake. Compared to normal (low fat) or high fat, high carbohydrate diets, obesity produced by a very high fat but low sugar diet resulted in increased sensitivity to both D1 and D2 receptor antagonism in reducing sucrose intake, but D2 receptor control of fructose intake was preserved. In contrast, rats fed a high-energy diet with a combination of high dietary fat and carbohydrate demonstrated enhanced D2 receptor regulation of fructose intake. Thus, it appears that dietary history may alter the development of dopamine deficits previously attributed to obesity in general. The present data also suggest that these particularities of dopamine plasticity may influence how certain carbohydrates, such as
fructose and sucrose, exert their rewarding effects. Such differences could explain some of
the variation in the success rates of different anti-obesity treatments and therapies. Further
studies are required to test the applicability of these findings to humans and investigate
underlying mechanisms.
Chapter 3.

Glucagon-like peptide-1 regulation of carbohydrate intake
is differentially affected by obesogenic diets

*This chapter includes results from the manuscript: Pritchett CE, Hajnal A. (2012). Glucagon-like peptide-1 regulation of carbohydrate intake is differentially affected by obesogenic diets. *Obesity, 20*(2), 313-317. See Appendix for published manuscript.

3.1. Introduction

With the escalating prevalence of high-energy diets, increased attention must be given to the physiological changes that follow chronic consumption of obesogenic diets leading to obesity. Obesogenic diets include those high in fat, carbohydrates or combinations of these macronutrients (Gibson, 1996). Such diets are readily consumed by both animals and humans and often lead to weight gain (Gibson, 1996; Levin and Dunn-Meynell, 2002). Such diet-induced obesity is associated with changes in a number of physiological signals (Hariri and Thibault, 2010; Madsen et al., 2010). One such signal is the gut hormone glucagon-like peptide-1 (GLP-1).

GLP-1 is released by enteroendocrine L cells of the ileum following ingestion of a meal (Drucker, 1998; Hayes et al., 2010; Strader and Woods, 2005). While GLP-1 is able to produce feelings of satiety in lean and obese humans following peripheral administration (Gutzwiller et al., 1999; Naslund et al., 1999), postprandial GLP-1 release has also been shown to be reduced in some obese humans and animals (Bojanowska, 2005; Ranganath et al., 1996). Normally, GLP-1 release results in a suppression of food intake (Drucker, 1998; Strader and Woods, 2005). Reports show that exogenous GLP-1, as well as the synthetic analog Exendin-4, also reduces food intake when administered systemically or directly into the brain in normal weight animals (Tang-Christensen et al., 1996; Turton et al., 1996). While less studied, others have also begun to investigate how GLP-1 influences aspects of...
sucrose consumption (Asarian et al., 1998). The effects of GLP-1 receptor activation in a model of diet-induced obesity, and its role in hedonically driven consumption (i.e., consumption driven by palatably of the food, rather than hunger), are not as well characterized. Differences due to various obesogenic diets also remain unknown, although the varying plasma GLP-1 levels of obese individuals indicate potential differences (Adam and Westerterp-Plantenga, 2005; Bojanowska, 2005; Holdstock et al., 2008; Morinigo et al., 2006; Ranganath et al., 1996). Such ambiguity points strongly to factors other than the presence of obesity alone in producing GLP-1 dysregulation. Therefore, we investigated how different obesogenic diets may influence the inhibitory effects on carbohydrate intake produced by activation of the GLP-1 receptor using the long-acting synthetic GLP-1 analog Exendin-4.

Obesogenic diets, such as high-sugar and high-fat diets, often produce hyperphagia leading to increased body weight and fat mass, and over time can eventually cause hyperinsulemia, hyperleptinemia, and insulin resistance (Madsen et al., 2010; Woods et al., 2004). Based on the macronutrient content and similarities between the obesogenic diet–fed rat and the obese human, these diet induced obesity models are considered applicable to human obesity (Madsen et al., 2010). On this basis, we used two obesogenic diets of varying macronutrient content to induce weight gain in rats. Obese rats and their lean counterparts were then tested in a one-bottle intake test of two sweet carbohydrate solutions with and without activation of GLP-1 receptors. We chose two common carbohydrates, sucrose and fructose, which are both preferred by rats and humans but have been shown to differ in their post-ingestive effects, including their rewarding properties. Specifically, whereas sucrose has been shown to produce conditioned effects based on both its taste and post-ingestive properties (Ackroff, 2008), fructose appears to exert behaviorally relevant stimulation.
exclusively by its taste and not by reinforcing post-ingestive effects (Sclafani and Ackroff, 1994). To our knowledge, changes in intake of these common carbohydrates following activation of GLP-1 receptors are yet unreported in obese rats. Furthermore, rats remained sated with *ad libitum* food available throughout the experiments to discern the possible role of GLP-1 in hedonic (palatability-driven) consumption, instead of consumption due to homeostatic regulation (hunger due to caloric deprivation).

### 3.2. Methods and Procedures

#### 3.2.1. Animals

Naive adult male Sprague-Dawley rats (Charles River, Wilmington, MA) weighing 250–275 g at the beginning of the study were housed in individual cages in a temperature-controlled vivarium and maintained on a 12:12-h light–dark cycle (lights on at 07:00). Animals were maintained on *ad libitum* standard laboratory chow (Chow; *n* = 8), a high-fat content, high-energy diet (HFHE; *n* = 11) or a high energy diet consisting of both fat and carbohydrate content (FCHE; *n* = 11). Animals were maintained on the diet for 28 weeks prior to experiments and throughout the testing period. During this time, rats received equal but limited exposure to sucrose and fructose in behavior-only test paradigms. Food and water were available *ad libitum* throughout testing.

#### 3.2.2. Diets, test solutions, and drugs

Standard laboratory chow (Teklad no. 2018, Somerville, NJ) was used as the control diet (3.4 kcal/g, 17% kcal from fat, 60% kcal from carbohydrates, and 23% kcal from protein). The high-energy diets were nutritionally complete diets obtained from Research Diets (New Brunswick, NJ). The high-fat high-energy diet (HFHE; Research Diets no. D12492) consisted of 5.24 kcal/g (60% kcal from fat, 20% kcal from carbohydrates, and 20% kcal.
from protein). The fat–carbohydrate high-energy diet (FCHE; Research Diets no. D12451) consisted of 4.73 kcal/g (45% kcal from fat, 35% kcal from carbohydrates, and 20% kcal from protein). Sucrose and fructose (Fisher-Scientific, Fair Lawn, NJ) were dissolved in filtered tap water (source identical to water available in home cages) and prepared no more than 24-h prior to testing. Exendin-4 was obtained from Tocris Biosciences (Ellisville, MO) and dissolved in sterile saline containing 1% albumin. Vehicle, i.e., saline containing 1% albumin, was used as the control solution.

3.2.3. Behavioral tests

To test the effects of GLP-1 on carbohydrate consumption, we used the synthetic analog Exendin-4 (0, 0.5, 1, 2, and 3 μg/kg; s.c.). Previous research has shown that doses within this range reduce chow intake in rats (Barrera et al., 2009; Bojanowska and Nowak, 2007). Whereas many studies have been carried out in food deprived rats, in our design the rats were fed ad libitum throughout the whole experiment, including the test phase. The rationale for that is the fact that an increasing number of calories consumed by the average US adult comes from high-sugar content beverages and snacks which are consumed independent of main meals, i.e., as treats (Archer et al., 2007; Briefel and Johnson, 2004). All tests were conducted in the middle of the light phase and in the home cage. Test solutions were administered via a single 100-ml bottle attached to the front of the home cage, with the spout extending into the home cage. Rats were trained to lick sucrose and fructose in this manner prior to Exendin-4 testing. Animals were treated with Exendin-4 10 min before 2-h access to 0.3 mol/l sucrose solution. Animals were given 4 days to recover, and then Exendin-4 was administered immediately prior to 2-h access to 0.4 mol/l fructose. These concentrations were chosen as they are readily consumed by rats and have been used extensively in the literature sen as they are readily consumed by rats and have been used extensively in the
literature (Baker et al., 2003; Hajnal et al., 2004). Amount consumed (in ml) was measured. A minimum of 2 days was given between injections.

3.2.4. Statistical analyses

Body weight data were analyzed using a two-way analysis of variance (ANOVA) with diet and dose as the independent variables, with separate ANOVAs conducted for body weight during sucrose and fructose testing periods.

Carbohydrate intake was measured as ml consumed. To evaluate differences in baseline intake, a two-way ANOVA was conducted on intake after vehicle (0 μg/kg) with diet group and carbohydrate as independent variables. The ANOVA revealed no effect of Diet (F(2,54)=2.8802, P=0.06) or Diet x Carbohydrate (F(2,54)=0.7096, P=0.50), but did reveal a significant difference on baseline intake of the two carbohydrates (F(2,54)=8.1669, P<0.01). Therefore, intake was converted to percent reduction from baseline (intake following dose X of Exendin-4/intake following 0 μg/kg Exendin-4 (27)) and is presented as mean ± s.e.m. Percent reduction was analyzed using a two-way ANOVA with Diet and Treatment (0, 0.5, 1, 2, or 3 μg/kg Exendin-4) as independent variables. Data were further analyzed for median inhibitory dose (ID50), calculated as previously described (Hajnal et al., 2007b) and compared as a function of Diet and Carbohydrate using Student’s t-tests. For all experiments, significant findings were further analyzed using Fischer’s least significant difference post hoc tests when appropriate. For all statistical analyses, the software Statistica (version 6.0, StatSoft, Tulsa OK) was used.

3.3. Results

3.3.1. Body weight

ANOVA revealed a consistent difference in body weight between Chow and obesogenic diet
groups throughout the experiment. For the sucrose test period, ANOVA revealed a significant effect of Diet ($F(2,162)=68.16, P<0.001$) but not Dose ($F(5,162)=0.06, P=0.998$) or Diet x Dose interaction ($F(10,162)=0.01, P=1.00$) on body weight. Post hoc analysis showed HFHE and FCHE rats weighed significantly more than Chow rats at each time point, while no significant differences were observed between obese groups (vs. Chow, HFHE: $P<0.001$; FCHE: $P<0.001$, all time points, Figure 3.1A).

ANOVA also revealed a significant effect of Diet ($F(2,162)=56.62, P<0.001$) but not Dose ($F(5,162)=0.02, P=0.999$) or Diet x Dose interaction ($F(10,162)=0.01, P=1.00$) on body weight throughout the fructose test period. Post hoc analysis again showed Chow rats had significantly lower body weight than HFHE or FCHE at each time point, but no significant differences were observed between obesogenic diet groups (vs. Chow, HFHE: $P<0.001$; FCHE: $P<0.01$, all time points, Figure 3.1B).
40

**Figure 3.1.** Body weight during pharmacological tests. Rats maintained on obesogenic diets for 28 weeks and throughout testing had significantly higher body weight than Chow-fed rats. There was no difference in body weight between HFHE or FCHE groups. (a) Average body weight by groups throughout the sucrose tests administered 24 h after no treatment (baseline weight), vehicle (0 μg/kg) or Exendin-4 treatment. (b) Body weight by group throughout the fructose test measured 24 h after no treatment, vehicle, or Exendin-4 treatment. *P < 0.01, **P < 0.001 compared to Chow at the same time point. FCHE, fat–carbohydrate high energy diet; HFHE, high-fat content, high energy diet.

3.3.2. Effects of GLP-1 receptor agonist on sucrose intake

A two-way ANOVA of normalized sucrose intake revealed significant effects of Diet \(F(2,135)=9.468, P<0.001\) and Dose \(F(4,135)=66.636, P<0.001\) but not a significant Diet x Dose interaction \(F(8,135)=0.990, P=0.45\) on percent reduction of baseline (Figure 3.2). Whereas all groups showed a dose-dependent reduction in sucrose intake, the effect was contingent upon diet conditions, with post hoc analysis showing overall sucrose intake was suppressed in HFHE rats to a lesser degree than FCHE \(P<0.01\) and Chow \(P<0.001\). Analysis of dose effects revealed that Exendin-4 significantly reduced sucrose intake in HFHE rats at 1 μg/kg and higher doses (1, 2, and 3 μg/kg; all \(P<0.001\)) while all doses
suppressed sucrose intake in FCHE rats (0.5 μg/kg: P<0.01; 1, 2, and 3 μg/kg: P<0.001). Chow rats showed reductions at 1 μg/kg and above (1, 2, and 3 μg/kg; all P<0.001).

Figure 3.2. Changes in sucrose intake following Exendin-4. Data are depicted as a reduction from baseline intake (i.e., intake following vehicle injection; set at 100% on y-axis). Sucrose intake was significantly reduced from baseline in all groups at 1, 2, and 3 μg/kg. However, Exendin-4 was less effective at suppressing sucrose intake in HFHE rats. **P < 0.01; ***P < 0.001 compared to 0 μg/kg Exendin-4. ##P < 0.01, ###P < 0.001 indicates diet group differences. FCHE, fat–carbohydrate high energy diet; HFHE, high-fat content, high energy diet.

3.3.3. Effects of GLP-1 receptor agonist on fructose intake

A two-way ANOVA of normalized fructose intake revealed significant effects of Diet (F(2,135)=14.329, P<0.001) and Dose (F(4,135)=25.549, P<0.001) but not a significant Diet x Dose interaction (F(8,135)=1.097, P=0.369; Figure 3.3). All groups showed a dose-dependent reduction in fructose intake, with differential sensitivity by different diet
conditions. Suppression of fructose intake in HFHE was blunted compared to FCHE (P<0.001) or Chow rats (P<0.001). Post hoc analyses of percent reduction from baseline revealed that only the highest doses (2 μg/kg: P<0.01; 3 μg/kg: P<0.001) reduced intake in HFHE rats. All doses significantly reduced intake in FCHE rats in a dose-dependent manner (0.5 μg/kg: P<0.01; 1, 2, and 3 μg/kg: P<0.001). Chow rats also showed a dose-dependent reduction in fructose intake following all Exendin-4 doses (0.5 μg/kg: P<0.01; 1 μg/kg: P<0.01; 2 and 3 μg/kg: P<0.001).

**Figure 3.3.** Changes in fructose intake following Exendin-4. Data are depicted as in Figure 2. Fructose intake was reduced in FCHE and Chow by all doses. Only the highest doses, 2 and 3 μg/kg Exendin-4, significantly reduced intake in HFHE rats, which were overall less sensitive to Exendin-4 than FCHE and Chow groups. **P < 0.01; ***P < 0.001 compared to 0 μg/kg Exendin-4. ###P < 0.001 indicates diet group differences. FCHE, fat–carbohydrate high energy diet; HFHE, high-fat content, high energy diet.
3.3.4. ID50

Calculation and analyses of the ID50 revealed significant differences in the sensitivity to Exendin-4 based on diet (Table 3.1). Calculation of the ID50 for both tests further supported the findings of the previous analysis and demonstrated that HFHE rats were the least sensitive of all groups to Exendin-4 treatment. For sucrose, independent t-tests revealed significant differences between HFHE and FCHE groups \( t(86)=2.99, P<0.01 \) and HFHE and Chow groups \( t(74)=2.34, P<0.05 \), but not between FCHE and Chow groups \( t(74)=0.061, P=0.952 \). Analysis of the ID50 for fructose again revealed significant differences between HFHE and FCHE groups \( t(86)=2.71, P<0.01 \) and HFHE and Chow groups \( t(74)=2.62, P<0.01 \) but not between FCHE and Chow groups \( t(74)=0.061, P=0.952 \).

<table>
<thead>
<tr>
<th>Diet effect on ID(_{50}) (μg/kg)</th>
<th>HFHE</th>
<th>FCHE</th>
<th>Chow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>3.3 ± 1.1**##</td>
<td>2.2 ± 0.3</td>
<td>2.5 ± 0.1</td>
</tr>
<tr>
<td>Fructose</td>
<td>3.7 ± 1.9***##</td>
<td>1.6 ± 0.3</td>
<td>1.5 ± 0.9</td>
</tr>
</tbody>
</table>

Table 3.1. Effectiveness of GLP-1 receptor stimulation by Exendin-4 as calculated by median inhibitory dose (ID50). The calculated doses at which intake is reduced by 50% of vehicle baseline (0 μg/kg) are depicted. **P < 0.01; compared to Chow, ##P < 0.01 compared to FCHE. FCHE, fat–carbohydrate high-energy diet; GLP, glucagon-like peptide; HFHE, high-fat content, high-energy diet.

3.4. Discussion

The present study assessed the sensitivity to GLP-1 receptor activation in two animal models of dietary obesity on suppressing intake of palatable carbohydrates. Extended exposure to the obesogenic diets resulted in significantly greater body weight in HFHE and FCHE groups.
compared to Chow controls, while no differences were observed between obesogenic diet groups (Figure 3.1). In contrast, we observed marked differences between diet groups following Exendin-4 treatment. HFHE rats were less sensitive overall to GLP-1 receptor activation, particularly when fructose was the test stimulus. Exendin-4 treatments did not significantly alter body weight measured 24 h after the injection, indicating GLP-1’s effects on carbohydrate intake were not likely due to a marked effect on homeostatic, energy-regulatory systems. These data support the notion that palatable carbohydrate intake above and beyond homeostatic needs can be regulated by GLP-1 receptor activation. Furthermore, this data demonstrate for the first time that GLP-1 signaling may be differentially altered in different models of dietary obesity, possibly explaining some of the incongruent findings on GLP-1 release in obese humans (Adam and Westerterp-Plantenga, 2005; Holdstock et al., 2008; Morinigo et al., 2006; Ranganath et al., 1996). We found that rats fed a high-energy diet from which the kilocalories came predominantly from high dietary fat content were markedly less sensitive to the inhibitory effects of Exendin-4 (Figures 3.2 and 3.3). On the other hand, rats on a high-energy diet consisting of moderately high dietary fat and carbohydrate content did not differ from lean controls, despite their significantly greater body weight (Figure 3.1). These findings were further supported by analyzing the inhibitory dose at which intake was reduced to 50% of baseline (ID50, Table 1). For both sucrose and fructose, HFHE rats required a higher ID50 than FCHE- or Chow-fed rats. These findings collectively suggest that a history of dietary fat intake, not obesity alone, may diminish GLP-1 signaling to curb carbohydrate intake.

Previous research has shown that Exendin-4 administered peripherally reduces chow intake in hungry rats (Tang-Christensen et al., 1996). Studies in lean and obese humans have also shown suppression of appetite and the intake of a balanced meal following GLP-1
administration (Gutzwiller et al., 1999; Naslund et al., 1999). Our data show that a GLP-1 receptor agonist can also potently reduce the intake of palatable carbohydrates in lean and obese rats when tested in a sated state. This is a novel observation and is relevant to understanding GLP-1’s role in hedonic-driven eating, i.e., intake that is independent of the drive due to actual energy deficits. Relevant to this are the reports from both human and animal studies demonstrating increased postprandial GLP-1 release following Roux-en-Y gastric bypass (Beckman et al.; Morinigo et al., 2006) and ileal interposition surgeries (Culnan et al., 2010; Strader et al., 2005), both of which are also known to reduce appetite despite restricted caloric intake. Furthermore, gastric bypass but not food restriction appears to reduce behavioral and neural taste functions for sweet taste in obese rats (Hajnal et al., 2010b). One potential explanation of these findings is that GLP-1 may directly engage the food reward system in addition to energy regulatory circuits. In fact, current research in our laboratory strongly suggests that GLP-1 released endogenously may modulate activity of dopamine neurons in the ventral tegmental area in vivo (unpublished data, See Chapter 4).

An additional riddle is the relationship between GLP-1 signaling, palatable carbohydrates, and obesity. We observed a blunted response in obese HFHE rats compared to chow controls. Such effects could be due to a reduction in endogenous GLP-1 release in obese HFHE rats. Recent research by Williams and colleagues (Williams et al., 2011) has shown that rats maintained for 4 weeks on a high-fat, high-energy diet identical to that used in the present study had lower fasting levels of active GLP-1 compared to rats fed a high-carbohydrate (70%), low-fat (10%) diet. In this study, similar to our study, the high-fat diet–fed rats also exhibited a blunted response to Exendin-4 treatment in 24-h food intake tests, compared to the low-fat, high-carbohydrate diet–fed group. Unfortunately, chow-fed controls were not available for comparison in that study. Previous research has also shown that GLP-1
secretion may be induced by both sucrose and fructose (Fukase et al., 1992; Gribble et al., 2003). Though not measured directly, these observations along with the study by Williams and colleagues collectively suggest that a reduction in endogenous GLP-1 secretion may result from extended exposure to high-fat diets, and this in turn could lead to impaired processing of taste (Martin et al., 2009). In addition to a plausible change in the regulation of peripheral taste information (Kokrashvili et al., 2009; Martin et al., 2009), GLP-1 may also alter central taste processing. In fact, central GLP-1 administration has been shown to reduce sham feeding of sucrose (Asarian et al., 1998). Changes to the behavioral sequence of sucrose ingestion following GLP-1 administration indicate that central GLP-1 may indeed regulate positive-feedback from a meal, and in turn, reduce intake of palatable foods by attenuating the perceived orosensory reward of the palatable food (Alhadeff et al., 2011; Asarian et al., 1998; Chelikani et al., 2005).

Taken together, one potential implication of these findings is that excessive consumption of high dietary fat may render anorexigenic gut-brain feedback less effective and in turn, increased stimulation by sweet taste on intake may remain unchecked. Thus, successful treatment of overweight and obesity may require a reduction of the desire to overconsume palatable and obesogenic foods, as well as restoration of diminished anorexigenic signals. A similar effect has been achieved by gastric bypass surgery, which increases postprandial GLP-1 response and also reduces appetite and sweet cravings (Hajnal et al., 2010; Holdstock et al., 2008; Morinigo et al., 2006). Improved understanding of the underlying mechanisms could help with developing an effective noninvasive treatment of obesity by reducing the drive to overconsume palatable foods. Furthermore, these findings caution that in the quest for identifying novel drug targets, conflicting responses may occur due to differences in the source of obesity, including macronutrient content of the diet.
Chapter 4.

Glucagon-like peptide-1 receptors expressed in the VTA: A potential target for curbing hedonic eating

* This chapter includes results from the manuscript: Pritchett CE, Toth, K, Abraham H, Hajnal, A. Glucagon-like peptide-1 receptors expressed in the VTA: A potential target for curbing hedonic eating. To be submitted to *Nature Neuroscience*, in final preparation.

4.1 Introduction

Glucagon-like peptide-1 (7-36) amide (GLP-1) is an incretin hormone released by nutrient sensing L cells following the intake of a meal to promote a healthy glycemic state (Drucker, 1998; Hayes et al., 2010). The peptide also exhibits anorexigenic properties inhibiting stomach acid secretion and gastric emptying, while also increasing satiety through receptors in the central nervous system (Drucker, 1998; Turton et al., 1996). GLP-1 receptor (GLP-1R) expression is widespread throughout the brain, and the central GLP-1 system has been shown to reduce intake of food and water in hungry rats (Drucker, 1998; Hayes et al., 2010; Turton et al., 1996). Administration of GLP-1 intravenously or into the cerebro-ventricular system also reduces sucrose intake in rats during sham-feeding procedures, indicating GLP-1 control of intake does not require the typical post-ingestive feedback signals (Asarian et al., 1998; Tang-Christensen et al., 1996). These and other reports demonstrate that GLP-1 is involved in the control of appetite, including intake of highly palatable foods such as sucrose. Such findings are also applicable to humans, as intravenous infusions of GLP-1 in hungry volunteers reduced meal duration, food intake and caloric intake, while subjects reported increased feelings of satiety with no adverse side effects (Gutzwiller et al., 1999).

While the satiety effects of GLP-1 have been widely investigated in hungry rodents and humans, there are far fewer investigations into the effects of GLP-1 on intake driven
solely by the rewarding value of the food. Reports indicate excess intake of highly palatable foods, that are often rich in fat and sucrose and eaten outside of normal meals, may be a key factor in the rise in rates of obesity (Levine et al., 2003b; Nguyen and El-Serag, 2010; Saris, 2003; Sorensen et al., 2003; Yeomans et al., 2004). Thus it has become increasingly important to understand the mechanisms underlying hedonically-driven eating, and recent reports have indicated GLP-1 may be a potential target for controlling such intake (Levine et al., 2003b; Nguyen and El-Serag, 2010; Saris, 2003; Sorensen et al., 2003; Yeomans et al., 2004).

Accordingly, to understand how GLP-1 influences hedonic consumption and perhaps the motivation to obtain and consume based on the hedonic value of the food, we conducted behavioral studies in sated rats with sucrose as the palatable food reward. We then targeted the ventral tegmental area (VTA) using immunohistochemistry to localize the expression of GLP-1Rs and electrophysiology to confirm the in vivo role of GLP-1Rs expressed within this reward-regulating nucleus.

4.2. Results

4.2.1. Central administration of GLP-1 reduces consummatory and operant responses to sucrose

The involvement of central GLP-1 in both unconditioned and conditioned aspects of hedonically driven sucrose intake was assessed in a series of experiments. These experiments also sought to localize neural regions in which GLP-1 may exert such effects. Sated male Sprague-Dawley rats were given brief access (30 min) to a 0.3M sucrose solution and intake was measured following central application of GLP-1. The data revealed that doses ranging from 2μg to 14μg GLP-1 administered directly into the fourth ventricle produced significant
reductions in the amount of sucrose consumed (Fig. 4.1a, n=9; one-way ANOVA, GLP-1 Dose: $F_{5,48} = 18.3997$, $p < 0.0001$). Indeed, at the highest dose tested, 14 μg of GLP-1 reduced sucrose intake by 50% or greater (Tukey’s HSD, 0.3 μg: $p = 0.1539$; 2 μg: $p < 0.001$, 6 μg: $p < 0.001$, 6 μg, $p < 0.001$, 14 μg, $p < 0.001$) compared to aCSF administration alone. However, when administered into the lateral ventricle, identical doses of GLP-1 were unable to elicit changes in sucrose intake (Fig. 4.1a inset, n=6; ANOVA: GLP-1 Dose: $F_{2,15} = 0.0963$, $p = 0.9087$).
GLP-1 (Infused into 4th ventricle)

b

Breakpoint (Mean ± SEM)

GLP-1 (Infused into 4th ventricle)

c

Active Spout Licks (Mean ± SEM)
GLP-1 (Infused into 4th ventricle)

**Figure 4.1.** GLP-1 is more effective at reducing sucrose intake when administered in the hindbrain. (a) Sucrose intake was reduced in lean, ad libitum chow-fed rats (n=9) following fourth ventricle administration of GLP-1 in a brief access (30 min) test. GLP-1 or vehicle (aCSF) was administered immediately prior to sucrose access and measured at 30 min (in mL). GLP-1 administered into the lateral ventricle (a, inset; n=6) did not produce similar reductions in sucrose consumption. (b) Sucrose-motivated behaviors were also reduced by GLP-1 applied to the fourth ventricle in lean, ad libitum chow-fed rats (n=8), as shown by reduction in the average breakpoint (operationally defined as the number of completed cycles, see Online Methods), while on a PR-10 schedule of reinforcement. This reduction was blocked by pre-administration of the GLP-1R antagonist Exendin-9 (Ex-9). (c) The number of licks made on the active spout was also depressed by central GLP-1 in the fourth ventricle only. (d) During the operant session, GLP-1 attenuated not only seeking behaviors for sucrose, but also actual sucrose intake when presented with the sucrose-containing spout. Mean ± s.e.m.; **P < 0.01, ***P < 0.001 compared to aCSF; # P < 0.05, ## P < 0.01 compared to 6.0 μg + aCSF.

After demonstrating that central GLP-1 can exert control over hedonic consumption, we investigated how similar treatments may influence the actual rewarding (incentive motivation) value of sucrose. Previous research has shown that food intake can be distinguished as two distinct phases (Berridge, 2009). During the appetitive stage, the organism uses seeking behaviors to locate an acceptable food source, which it can then
consume, initiating the consummatory phase (Craig, 1917). In an experimental design allowing for separation of the two phases of eating, naïve, ad libitum-fed male Sprague-Dawley rats (n=8) were trained to lick an empty spout on a progressive ratio-10 (PR-10) operant schedule of reinforcement (appetitive behavior) in order to receive brief access (15 seconds) to the same (0.3M) concentration of sucrose previously tested (consummatory phase) (See Online Methods for more details). We found that 6 μg GLP-1 administered into the fourth ventricle produced significant reductions in sucrose-seeking behavior of sated rats. While on the rigorous PR-10 schedule, GLP-1 lowered the number of completed cycles (Fig 1b), operationally defined as the “breakpoint” (GLP-1 Dose: $F_{5,42} = 4.0010$, p < 0.01), GLP-1 also attenuated reward-seeking behavior as measured by licks made on the active spout to work towards a sucrose reward (Fig. 4.1c, $F_{5,42} = 2.5209$, p < 0.05). Along with reducing appetitive responses, we also saw reductions in sucrose consumption following GLP-1R activation (Fig. 1d, $F_{5,42} = 2.7240$, p < 0.05). In contrast, the number of inactive licks were not affected by GLP-1 (Table 1), indicating the effects of GLP-1 were not due to depressing general locomotor activity. All observed effects were then blocked by pretreatment with the GLP-1R antagonist Exendin-3 (9-39)amide (Ex-9), in a dose that alone did not alter behavior (Fig. 4.1b, c, d).

<table>
<thead>
<tr>
<th>GLP-1 (Infused into fourth ventricle; Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>aCSF</td>
</tr>
<tr>
<td>Inactive Licks</td>
</tr>
<tr>
<td>Latency to Lick Sucrose Spout</td>
</tr>
<tr>
<td>Latency to Lick Active Spout</td>
</tr>
</tbody>
</table>

Table 4.1. Measures of operant performance for a sucrose reward following fourth ventricle administration of GLP-1.
Because forebrain areas also express GLP-1 receptors (Alvarez et al., 1996) and multiple sites of action may modulate different aspects of food intake (e.g. affecting brain circuitries involved in energy regulatory, learning, or cognitive aspects), a separate group of animals were tested to determine if the influence of GLP-1 would also be effective when infused into the lateral ventricle (n=11). When tested on a PR-10 schedule, the same 6 μg dose of GLP-1 that reduced operant responses to procure or consume sucrose reward when administered in the fourth ventricle did not alter the same measures when administered in the lateral ventricle (Table 4.2).

<table>
<thead>
<tr>
<th>GLP-1 (Infused into lateral ventricle; Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>aCSF</td>
</tr>
<tr>
<td>-------</td>
</tr>
<tr>
<td>558.8 ± 68.4</td>
</tr>
<tr>
<td>487.2 ± 75.0</td>
</tr>
<tr>
<td>6.1 ± 2.8</td>
</tr>
<tr>
<td>1.08 ± 0.05</td>
</tr>
<tr>
<td>48.8 ± 6.4</td>
</tr>
</tbody>
</table>

Table 4.2. No changes were observed in operant performance for a sucrose reward following lateral ventricle administration of GLP-1.

To ensure that the lack of an effect was not due to the doses chosen, we also tested animals following 14 and 20 μg GLP-1. However, none of the GLP-1 doses reduced the breakpoint (ANOVA, GLP-1 Dose: $F_{5,60} = 0.2587, p = 0.9338$), lowered the number of licks made on the active spout ($F_{5,60} = 0.41027, p = 0.8398$), or reduced the number of licks made on the spout containing sucrose ($F_{5,60} = 0.1744, p = 0.9711$). Thus, it appears that GLP-1 infused into the lateral ventricle is insufficient to alter sucrose intake or the motivation to work for sucrose reward (Table 4.2). Overall, these findings strongly suggest that in this rat model, GLP-1 receptors in the brainstem and not in the forebrain may modulate unconditioned or
conditioned sucrose intake via modulating its rewarding value.

4.2.2. GLP-1 within the Ventral Tegmental Area Reduces Sucrose Intake and Sucrose Conditioned Behaviors

Previous literature has clearly demonstrated the role of the mesolimbic, or central reward system, in motivated behaviors such as the consumption of drugs or natural rewards like sugar (Berridge, 2009; Berridge et al., 2010; Mark et al., 2011; Wise, 2006). One mesolimbic area commonly found to influence such behaviors is the ventral tegmental area (VTA), a dopamine rich nucleus which projects to a number of brain regions shown to be involved in reward-related behaviors (Berridge et al., 2010; Gearhardt et al., 2011; Volkow et al., 2008). Localization of the GLP-1 effect to the hindbrain led us to our next set of experiments in which we directly targeted a single nucleus potentially involved. Direct infusion of various doses of GLP-1 were applied to the VTA prior to access to sucrose (Fig. 4.2a, n=10). Similar to the findings from infusions in the fourth ventricle, we found GLP-1 dose-dependently reduced sucrose intake compared to aCSF (Fig. 2b, $F_{5,54} = 4.0225$, $p < 0.01$). While pre-treatment with the GLP-1 receptor antagonist Ex-9 did not significantly increase intake alone, it did completely block the reduction in sucrose intake seen following the most effective dose of GLP-1 (Fig. 4.2b; $p<0.05$ compared to 1.0 μg GLP-1).
**Figure 4.2.** GLP-1 reduces sucrose intake and appetitive behaviors when applied to the ventral tegmental area. (a) Schematic representation of injector placement within the ventral tegmental area (VTA). (b) Sucrose intake by lean, *ad libitum* chow-fed rats (n=10) was dose-dependently reduced by GLP-1 administered into the VTA immediately prior to brief (30 min) access to sucrose. Mean ± s.e.m.; * P < 0.01, **P < 0.01, ***P < 0.001 compared to aCSF; # P < 0.05 compared to 1.0 µg + aCSF.

Once establishing the VTA as an area in which GLP-1 could produce significant changes in unconditioned sucrose intake, we next investigated the role of VTA GLP-1Rs on operant responses for sucrose. As in the previous experiments, naïve *ad libitum* male Sprague-Dawley rats (n=6) were trained to lick an empty spout on a PR-10 operant schedule of reinforcement, and received a sucrose (0.3M) reward upon completion of the requirements. Animals were fitted with bilateral cannula aimed at the VTA (Fig. 4.2a) prior to training, and GLP-1 was administered directly into the VTA immediately before PR10 sessions. GLP-1 produced significant, dose-dependent reductions (Fig. 4.3a, Fig. 4.3b) in how willing the animals were to work for the 0.3M sucrose reward (breakpoints: $F_{4,23} = 3.0138$, p < 0.05; active spout licks: $F_{4,23} = 4.0593$, p < 0.05). Intra-VTA GLP-1 also attenuated consummatory responses, as the amount of sucrose consumed on the reward spout was also reduced in a dose-dependent manner (Fig. 4.3c, $F_{4,23} = 2.9705$, p < 0.05).

<table>
<thead>
<tr>
<th>GLP-1 (Infused into fourth ventricle; Mean ± SEM)</th>
<th>aCSF</th>
<th>6 µg</th>
<th>14 µg</th>
<th>20 µg Ex-9 + 6 µg</th>
<th>20 µg Ex-9 + aCSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inactive Licks</td>
<td>16.7 ± 4.0</td>
<td>6.7 ± 1.8</td>
<td>4.5 ± 2.0</td>
<td>12.4 ± 4.2</td>
<td>11.4 ± 4.1</td>
</tr>
<tr>
<td>Latency to Lick Sucrose Spout</td>
<td>1.26 ± 0.09</td>
<td>1.32 ± 0.07</td>
<td>1.25 ± 0.08</td>
<td>1.31 ± 0.13</td>
<td>1.36 ± 0.10</td>
</tr>
<tr>
<td>Latency to Lick Active Spout</td>
<td>28.8 ± 3.4</td>
<td>25.4 ± 4.8</td>
<td>23.9 ± 5.2</td>
<td>30.4 ± 7.3</td>
<td>30.9 ± 5.2</td>
</tr>
</tbody>
</table>

**Table 4.3.** Operant performance behaviors for a sucrose reward following GLP-1 administered into the central tegmental area.
Figure 4.3. Sucrose-motivated seeking and consumption is reduced by GLP-1 in the VTA. (a) Lean, ad libitum chow-fed rats (n=6) were trained to work for and receive a sucrose reward on a PR-10 schedule (see Online Methods) showed reductions in the number of completed cycles (breakpoint) when tested immediately after intra-VTA administration of GLP-1. (b) The number of licks made on the active spout to work towards a sucrose reward was also reduced by GLP-1 in the VTA. (c) Sucrose intake was also dose-dependently reduced, albeit less potently, when GLP-1 was administered in the VTA. Mean ± s.e.m.; * P < 0.01, **P < 0.01, ***P < 0.001 compared to aCSF.

4.2.3. Dopamine neurons in the VTA express GLP-1 receptors

To follow up on the findings with functional effects by GLP-1 on dopamine-dependent behaviors, immunohistochemistry was used to determine neural localization of GLP-1Rs within the VTA. We found that neurons expressing the GLP-1R were detectable throughout the VTA. Most of the GLP-1R-immunoreactive neurons exhibited an elongated large nucleus and a bipolar cytoplasm (Fig. 4.4a). Approximately half of the GLP-1R-positive neurons were found to be dopaminergic, co-expressing the enzyme essential for dopamine synthesis,
tyrosine hydroxylase (TH), and nearly half of the TH-positive cells also co-expressed GLP-1R (Fig. 4.4, Table 4.4). Indeed, in the VTA, GLP-1Rs were found to be colocalized with TH in 49.8-59.5% (Mean ± SEM: 54.4 ± 3.50%) of neurons in the more ventral region of the VTA, and 51.8-60.2% (56.0 ± 3.15%) of neurons within the dorsal VTA (Table 4.4). However, not all dopaminergic neurons expressed GLP-1Rs, with little variability between the ventral and dorsal regions. Within the ventral VTA, 35.9-47.6% (41.9 ± 4.37%) of TH-immunoreactive neurons expressed GLP-1R and 41.7-48.8% (43.7 ± 2.93%) of TH-immunoreactive neurons expressed GLP-1R in the more dorsal region of the VTA (Table 4.4).
When investigating where the highest rate of co-localization of GLP-1R and TH positive neurons occurred, we found the difference in coexpression of GLP-1Rs and TH along the rostro-caudal extension to be negligible within the dorsal VTA (Table 4.4). However, there was a difference in the ventral VTA, with the highest rate of co-localization being observed in rostral region, at the 5.2-5.3 antero-posterior coordinates measured from the Bregma. Accordingly, we chose to target this area with the following electrophysiology investigations. In this region, 59.5% of the neurons were TH positive, and 47.6% of TH-
immunoreactive neurons also expressed GLP-1Rs (Table 4.4). In the more caudal regions, both the rate of GLP-1R-expressing neurons that contained TH, and the proportion of TH-immunoreactive neurons co-expressing GLP-1Rs were lower than in the most rostral area examined (Table 4.4).

<table>
<thead>
<tr>
<th>Area coordinates</th>
<th>Percentage of TH and GLP-1R coexpressing cells of GLP-1R positive cells</th>
<th>Percentage of TH and GLP-1R coexpressing cells of TH-positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP 5.2-5.3</td>
<td>59.5% (215/361)</td>
<td>47.6% (215/451)</td>
</tr>
<tr>
<td>ML 0.8-1.0</td>
<td>53.2% (239/449)</td>
<td>35.9% (239/644)</td>
</tr>
<tr>
<td>DV 8.4-8.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AP 5.4-5.5</td>
<td>55.1% (195/354)</td>
<td>44.1% (195/442)</td>
</tr>
<tr>
<td>ML 0.6-0.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DV 8.4-8.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AP 5.6-5.7</td>
<td>49.8% (132/265)</td>
<td>40.1% (132/323)</td>
</tr>
<tr>
<td>ML 0.5-0.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DV 8.3-8.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AP 5.8-5.9</td>
<td>54.4 ± 3.50</td>
<td>41.9± 4.37</td>
</tr>
<tr>
<td>ML 0.3-0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DV 8.3-8.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.4. Summary of the rate of co-expression of GLP-1R and TH in the medial and lateral VTA in different rostro-caudal extension. Abbreviations: AP, antero-posterior; ML, medio-lateral; DV, dorso-ventral.

This localization of GLP-1Rs directly upon dopamine neurons throughout the VTA could be one mechanism through which GLP-1 exerts behavioral effects. However, not all
GLP-1Rs were colocalized with TH-positive neurons, thus other neuronal types within the VTA must also express GLP-1Rs. Consequently, we sought to characterize other potential neural interactions through which GLP-1 may be exerting its effects.

Another prominent type of neuron within the VTA are GABAergic interneurons, which act as important modulators of dopamine neurons (Narayanan et al., 2010; Oades and Halliday, 1987). Therefore, immunohistochemistry was performed using antibody against GAD65/67 to detect the enzyme responsible for GABA synthesis. These results (Fig. 4.4, Table 4.4) show that within the rostral area of the ventral VTA, a subset of GLP-1R expressing neurons co-express GAD65/67, indicating that the GLP-1R is also expressed by these GABAergic interneurons. However, a portion of GLP-1R expressing neurons did not express either TH or GAD65/67.

4.2.4. GLP-1 alters the firing rate of dopaminergic neurons within the VTA

Because we found the largest number of GLP-1-expressing dopamine neurons within the rostral portion of the ventromedial VTA, we targeted this area to determine how both exogenous and endogenous GLP-1 signaling may influence the firing rates of dopamine neurons. To do so, single-unit extracellular recordings and microiontophoretic application of agonists or antagonists were used (See Online Methods). We recorded and analyzed the activity of 121 dopamine neurons in the VTA from 102 recording sites in 12 naïve Sprague-Dawley male rats maintained on isoflurane anesthesia during the recording session (Fig. 4.5a). The average basal firing rate was 1.95 ± 0.16 spikes/sec. Neurons were identified using well-established criteria, i.e., broad, frequently triphasic extracellular action potentials with a duration of more than 2 ms and a relatively slow firing rate of <10 Hz. We also measured aversive stimuli-induced inhibition of individual neuronal activity using foot pinch for further verification (Missale et al., 1998; Ungless et al., 2004b).
Total number of recorded VTA Dopamine neurons n=121

GLP-1 responsive neurons
n=69/121 (57%)

GLP-1 unresponsive neurons
n=52/121 (43%)

GLP-1 facilitated neurons
n=47/69 (68%)

GLP-1 inhibited neurons
n=22/69 (32%)
Figure 4.5. Electrophysiological responses of VTA dopamine neurons to GLP-1. (a) Left: Schematic representation of the histological localization of the 121 recording areas in the VTA. Right: Representative image of VTA recording site using cresyl violet staining. (b) Distribution of recorded neurons change in firing, based on the effects of GLP-1 application (through direct microiontophoretic application to the neuron, see Online Methods) (c) Average of neural effects expressed as a relative change to baseline firing activity (the spontaneous firing recorded prior to drug application). (d-f) Representative recordings of individual neurons responding to GLP-1, Exendin-9, and Bicuculline. Bottom panels represent the analog trace (in mV) of the recorded neuron. Top panels represent the averaged neuronal activity shown in number of spikes per second, with the trace of the representative neuron shown in the top right of the panel. (d) A recorded neuron exhibiting an excitatory effect by GLP-1. The same neuron was inhibited by application of the GLP-1 receptor antagonist Exendin-9 (Ex-9), suggesting tonic control through endogenous GLP-1. (e) A recorded neuron showing strong inhibition of activity following GLP-1 application. Co-application of Ex-9
with GLP-1 blocked the effect seen by GLP-1 alone. (f) A representative recording demonstrating an excitatory response to GLP-1 and blockade of that response with co-application of the GABA antagonist Bicuculline (BCC). The neuron then slowly recovers from an acute phase of insensitivity to GLP-1 after the BCC treatment. Mean ± s.e.m.; * P < 0.05.

Once dopaminergic neurons were identified and stable baseline responding was observed, GLP-1 and Ex-9 were applied alone or in combination, to test both the effects of exogenous (GLP-1 alone) and endogenous GLP-1 (Ex-9 alone) and to verify that changes induced by GLP-1 are mediated through GLP-1 receptor specific mechanisms (Ex-9 + GLP-1). The breakdown of responses to exogenous GLP-1 is shown in Figure 4.5b. Along with GLP-1 and the GLP-1R antagonist Ex-9, microiontophoretic application of other compounds was used for further identification and to test the interactions between GLP-1 and neurochemicals inherent to the VTA. These tests included microiontophoretic ejection of bicuculline methiodide (BCC), the selective post synaptic GABA_A receptor antagonist, the selective D2R agonist Quinpirole (QP), and L-glutamic acid (Glutamate) or γ-aminobutyric acid (GABA) as excitatory and inhibitory controls, respectively (See Online Methods).

All of the 121 dopamine neurons were tested with microiontophoretic application of the peptide GLP-1, and we observed a significant influence by GLP-1 on neuronal responses. Figures 4.5b and 4.5c depicts the distribution of responses to GLP-1, with more than half of dopamine neurons responding to GLP-1 (n=69, p<0.003). Most of these neurons (68%) exhibited a dramatic increase in firing rate, almost twice that of baseline firing (n=47, Student t test, \( t_{46} = 4.398, p < 0.0001, +1.568 \pm 0.36 \) spikes/sec, Fig. 4.5c). Of the remaining 32% of these GLP-1 sensitive dopamine neurons, application of GLP-1 inhibited the firing rate significantly (n=22, \( t_{21} = 5.650, p<0.0001, -0.840 \pm 0.15 \) spike/sec, Fig. 4.5c). A typical neuronal responding to GLP-1 and Ex-9 is shown in Figure 4.5d.

To further verify the specific effects of GLP-1, we altered the applied GLP-1 ‘dose’
by modification of the microiontophoretic current intensity. This allowed us to investigate dose dependent effects of GLP-1 in a subset of GLP-1-excited \((n=8)\) and GLP-1-inhibited \((n=4)\) dopamine neurons. In the GLP-1 excited neurons, changing the ejection current from 50nA to 100nA increased the excitatory effect of GLP-1 from \(2.39 \pm 0.56\) spikes/sec to \(3.39 \pm 0.55\) spikes/sec (vs. basal firing rate of \(1.31 \pm 0.31\) spike/sec.). We saw no significant differences in GLP-1 inhibited neurons with higher doses of GLP-1. This observation may be interpreted as a ‘floor-effect’, i.e. a resulting from the close apposition of the infusion channels with the recording electrode providing high local concentrations of GLP-1 even at the lowest tested dose sufficient to reduce the \textit{ab ovo} slow basal firing of the dopamine neurons.

**4.2.5. Exendin-3 (9-39) amide alters the firing rates of VTA dopamine neurons**

We administered the GLP-1R antagonist Ex-9 to 72% of GLP-1 responsive dopamine neurons \((50\text{ of }69\text{ neurons})\). When applied alone, Ex-9 significantly altered the firing rate of 68% of tested neurons \((n=34, \text{ANOVA}, F_{3,64} = 2.752, p < 0.05)\). Of these, 25 neurons were inhibited \((t_{24} = 3.800, p < 0.001, -1.590 \pm 0.42 \text{ spikes/sec, Fig. 4.5c, d, e})\) and 9 neurons were facilitated \((t_{8} = 3.706, p < 0.006, +1.357 \pm 0.37 \text{ spikes/sec, Fig. 4.5c, d, e})\) by Ex-9 alone. These observations suggest that endogenous GLP-1 may exert tonic control on the dopamine neurons or neurons within the local circuitry of the VTA, and these effects appear to more commonly be tonic excitation, with a small number of neurons under tonic inhibition by GLP-1, as observed by the changes from Ex-9 application. Such effects may dependent upon the pre- or post-synaptic location of the GLP-1 receptors and indicate there may be two distinct types of dopamine neurons, GLP-1R expressing and GLP-1R non-expressing. These observations are supported by the immunohistochemical results indicated only half of neurons expressing markers for dopamine co-expressed GLP-1Rs.
To determine if the observed electrophysiological effects of GLP-1 are mediated by GLP-1R activation on the cell surface, Ex-9 and GLP-1 co-application was performed on 23 of the 69 GLP-1 responsive dopamine neurons. Paired t-tests comparing pre- vs. poststimulus neuronal responses demonstrated that co-microinjection of Ex-9 abolished the effect of GLP-1 in all tested neurons (n=23, $t_{22} = 0.4496$, NS, Fig 4.5c, d, e). These data strongly indicate that the effects of GLP-1 within the VTA are receptor-dependent, despite the nature of the change of neural firing.

4.2.6. GLP-1 induced excitability of dopamine neurons is mediated by GABA

Based on the immunohistochemistry study demonstrating the presence of GLP-1Rs on GABAergic neurons in the VTA, we also examined the effects of GABAergic interactions with GLP-1 on dopamine neurons. To do so, the postsynaptic GABA_A receptor antagonist Bicuculline (BCC) was applied in combination with GLP-1 application. Using BCC, we were able to isolate the effects of GABA inputs on the recorded dopamine neurons with respect to GLP-1 responses. We applied BCC 30 sec before, or simultaneously with GLP-1 microiontophoresis (Fig. 4.5c, f). The BCC pre-and co-application was performed in 19 dopamine neurons previously identified as GLP-1-facilitated neurons. When pre-administered BCC for this relatively short time we did not observe any alteration in basal firing rate (1.425 ± 0.57 spikes/sec). In contrast, we found pre-application of BCC prevented the excitatory effect of GLP-1 in 17 out of 19 neurons (89%, paired t-test: $t_{16} = 1.215$, NS). These findings are not surprising as a consequence of the complex local regulation of VTA dopamine neurons. The ability of BCC to block GLP-1’s excitatory effect suggests that the direct excitatory effects of GLP-1 on dopamine neurons may be highly dependent upon continuous GABA input to the dopamine neuron. Indeed, the lack of a GLP-1 effect following BCC application indicates a constant GABAergic tone may even be necessary for
the excitation effects of GLP-1. Similar effects have been observed in the case of insulin signaling (Kovacs and Hajnal, 2009; Williams, 2008)

<table>
<thead>
<tr>
<th>Neural Responsiveness</th>
<th>Avg firing rate before drug application (over 30 sec)</th>
<th>Avg firing rate after drug application (over 30 sec)</th>
<th>Number of Neurons</th>
<th>Paired t-test P&lt;0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non GLP-1 Responsive</td>
<td>1.38±0.28</td>
<td>1.83±0.26</td>
<td>52</td>
<td>NS</td>
</tr>
<tr>
<td>GLP-1 excitation</td>
<td>1.43±0.22</td>
<td>3.00±0.46</td>
<td>47</td>
<td>*</td>
</tr>
<tr>
<td>GLP-1 inhibition</td>
<td>1.93±0.41</td>
<td>1.09±0.38</td>
<td>22</td>
<td>*</td>
</tr>
<tr>
<td>Non Ex-9 responsive</td>
<td>1.76±0.5</td>
<td>1.81±0.49</td>
<td>16</td>
<td>NS</td>
</tr>
<tr>
<td>Ex-9 excitation</td>
<td>0.88±0.19</td>
<td>2.24±0.49</td>
<td>9</td>
<td>*</td>
</tr>
<tr>
<td>Ex-9 excitation in GLP-1 facilitated neurons</td>
<td>1.83±0.33</td>
<td>2.73±0.71</td>
<td>4</td>
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<tr>
<td>Ex-9 excitation in GLP-1 inhibited neurons</td>
<td>0.58±0.24</td>
<td>1.21±0.32</td>
<td>5</td>
<td>*</td>
</tr>
<tr>
<td>Ex-9 inhibition</td>
<td>3.11±0.68</td>
<td>1.52±0.34</td>
<td>25</td>
<td>*</td>
</tr>
<tr>
<td>Ex-9 inhibition in GLP-1 facilitated neurons</td>
<td>3.41±0.84</td>
<td>1.68±0.42</td>
<td>20</td>
<td>*</td>
</tr>
<tr>
<td>Ex-9 inhibition among GLP-1 inhibited neurons</td>
<td>2.13±0.64</td>
<td>0.99±0.40</td>
<td>5</td>
<td>*</td>
</tr>
<tr>
<td>Ex-9 + GLP-1</td>
<td>1.03±0.26</td>
<td>1.08±0.29</td>
<td>23</td>
<td>NS</td>
</tr>
<tr>
<td>Non QP responsive</td>
<td>1.89±0.38</td>
<td>1.88±0.37</td>
<td>12</td>
<td>NS</td>
</tr>
<tr>
<td>QP Inhibition</td>
<td>2.36±0.68</td>
<td>0.99±0.28</td>
<td>21</td>
<td>*</td>
</tr>
<tr>
<td>QP inhibition in GLP-1 excited neurons</td>
<td>2.58±0.94</td>
<td>1.08±0.39</td>
<td>15</td>
<td>*</td>
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<tr>
<td>QP inhibition in GLP-1 inhibited neurons</td>
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<td>0.78±0.22</td>
<td>6</td>
<td>NS</td>
</tr>
<tr>
<td>QP excitation</td>
<td>4.44±1.08</td>
<td>7.71±1.52</td>
<td>15</td>
<td>*</td>
</tr>
<tr>
<td>QP excitation in GLP-1 excited neurons</td>
<td>4.99±1.26</td>
<td>8.68±1.94</td>
<td>10</td>
<td>*</td>
</tr>
<tr>
<td>QP excitation in GLP-1 inhibited neurons</td>
<td>3.92±2.20</td>
<td>5.78±2.46</td>
<td>5</td>
<td>NS</td>
</tr>
<tr>
<td>BCC+ GLP-1</td>
<td>1.43±0.57</td>
<td>1.54±0.61</td>
<td>17</td>
<td>*</td>
</tr>
</tbody>
</table>

Table 4.5. Summary of neuronal responsiveness to application of different neurochemicals.

4.2.7. Characterization of the recorded neurons for D2 receptor expression

To further characterize the recorded neurons for expression of D2 dopamine receptors, we applied iontophoretically the selective D2R agonist Quinpirole (QP). Quinpirole was applied to 48 electrophysiologically identified dopamine neurons from the rostral aspect of the VTA, and in all cases generated a significant effect (Fig. 4.6c; \( F_{5,90} = 8.963, p < 0.0001 \)).

Comparing 30 sec pre- vs. poststimulus firing rates using a paired t-test, QP produced no effect in 12 neurons (\( t_{11} = 0.081, \) NS), significant inhibitory effects in 21 dopamine neurons (\( t_{20} = 3.039, p < 0.007 \), and significant excitation of 15 dopamine neurons (\( t_{14} = \))
4.225, p < 0.0008). Further analyzing these neuronal subpopulations, it is notable that the basal frequency of QP excited dopamine neurons was twice as high (4.44 ± 1.08 spikes/sec) compared to the basal firing rate of QP inhibited dopamine neurons (2.30 ± 0.68 spikes/sec) suggesting a possible state dependent effect of QP, likely related to the expression of D2Rs.

4.2.8. Characterization of our neuronal population using GABA or Glutamate

During recording sessions, the general electrophysiological responsiveness of each neuron was characterized by application of GABA as an inhibitory control and/or ejection of glutamate as an excitatory control. The evaluation of neuronal responsiveness was based on a single application of either GABA or glutamate, because the former could critically inhibit the originally slow dopamine neurons, while the latter could be neurotoxic. In sum, all the examined 121 DA neurons responded to either GABA or glutamate microiontophoresis.

4.3. Discussion

Recently, increased attention has focused on GLP-1 as a peptide with an important role in feeding regulation, making it a potential as a pharmacological target to curb cravings, excess intake, and thus dietary obesity (Bojanowska, 2005; Drucker, 1998; Hayes et al., 2010; Moran and Dailey, 2009; Murphy and Bloom, 2004; Suzuki et al., 2010). Since central GLP-1 was first shown to influence food intake, many studies have explored where and how it may exert such actions, finding that GLP-1 receptors are located throughout the brain (Goke et al., 1995; Turton et al., 1996). These areas included sites controlling motivational and energy homeostasis such as various regions of the hypothalamus (including arcuate, paraventricular and lateral hypothalamic nuclei), as well as sites involved in reward such as the ventral tegmental area, the nucleus accumbens and amygdala. These later discoveries form the theoretical stage for the notion that central GLP-1 receptors may also control non-
homeostatic feeding (Dunphy et al., 1998). While the ventral tegmental area and associated mesolimbic dopamine system have long been implicated in reward-driven feeding (Berridge and Robinson, 1998; Berridge et al., 2010; Johnson and Kenny, 2010; Kelley and Berridge, 2002), only recently has evidence emerged that central neurons producing GLP-1 project from the nucleus of the solitary tract to the nucleus accumbens and the VTA and that direct application of GLP-1 agonists to the VTA alter feeding behaviors (Alhadeff et al., 2012). Thus, a novel “third mechanism” (in addition to vagal, and trans-BBB GLP-1 effects) utilizing GLP-1 produced within the brain must be considered in the pharmacological studies. While such studies have begun to highlight the potential importance of GLP-1 in reward-driven feeding, the specific interactions between GLP-1 and dopamine neurons in the VTA remained to be elucidated. The present study verified the hindbrain, and pointed to the VTA specifically, as sites that produce behavioral changes in sucrose intake and incentive motivation when working for a sucrose reward. In addition, our histological study confirmed the presence of GLP-1 receptors on VTA dopamine and GABA neurons, and demonstrated functional activity of these receptor showing that GLP-1 applied directly to dopamine neurons in the VTA alters the neuronal firing rate.

Initially, this study used behavioral studies to identify brain areas in which GLP-1 could reduce sucrose intake driven by palatability and not by a state of depletion and where GLP-1 exerted actions on operant responses for sucrose when presented as a reward. The results demonstrate the ability and efficacy of GLP-1 to reduce sucrose intake in animals never food deprived, indicating the hindbrain, and at least one of their constituents, the VTA is a primary site where such unconditioned, hedonically-driven consumption may be regulated by GLP-1 of either central or peripheral origin. (Fig. 4.3 & 4.4). Although this study did not investigate other motivated behaviors, it appeared that the observed effects
were specific to sucrose intake and sucrose-motivated behaviors because GLP-1 in the VTA did not alter general locomotor activity (Fig. 4.1, Table 4.1). These behavioral results are backed by immunohistochemistry and electrophysiology, supporting the conclusion that GLP-1 in the VTA has the necessary anatomical and functional machineries to bring about changes in this particular behavior (sucrose seeking). An additional uniform finding of the behavioral and electrophysiological tests was the discovery that GLP-1 receptors exert a tonic control on DA neuron firing implied from the disinhibitory effect by the GLP-1 receptor antagonist, Ex-9. Overall the data sheds new light on the interactions between GLP-1 and neurons in the mesolimbic dopamine system, which extends beyond dopamine neurons. The presence of GLP-1 receptors on both dopamine neurons and perhaps GABAergic interneurons in the VTA was established using immunohistochemistry (Fig. 4.4). The ability of GLP-1 to change the firing rates of intra-VTA dopamine neurons was not only demonstrated using direct application of GLP-1 to dopamine neurons (Fig. 4.5) but also assessed in context of manipulation of GABA input (Fig. 4.5). Indeed, it cannot be excluded that that dopamine neurons within the VTA may be under the regulation of both pathways and hence sensitive to GLP-1 through direct and indirect mechanisms. Although the focus of the present study was on the VTA, the somatodendritic unit of the dopamine neurons, the findings do not mitigate a potential role for GLP-1 in the terminal regions of the mesolimbic/mesocortical projections. Indeed, recent studies have shown that synthetic agonists such as Exendin-4 can influence behavior not only when administered directly into the VTA (Dickson et al., 2012) but also when injected in the nucleus accumbens (NAcc), particularly the NAcc core (Alhadeff et al., 2012; Dickson et al., 2012; Dossat et al., 2011). Furthermore, these data suggest that projections from the VTA to the NAcc core may be highly relevant in the control of food intake (Alhadeff et al., 2012), and may be controlled by
GLP-1 through both pre-, and postsynaptic mechanisms.

As the rates of obesity continue to rise, it is clear that understanding how the brain responds to palatable foods, and the mechanisms that control such hedonically-driven intake, are crucial to the success of obesity interventions (Nguyen and El-Serag, 2010; Oades and Halliday, 1987; Pelchat, 2009; Sorensen et al., 2003). The present studies clearly demonstrates the need and importance in understanding the physiological mechanisms underlying GLP-1’s actions on reward-related behaviors with special focus on the neuromodulatory role of GLP-1. Future studies are warranted to reveal the intracellular mechanisms by which GLP-1R stimulation may result in various effects and interactions with other regulatory and transmitter systems, and also to demonstrate transferability of the finding to human.

4.4. Online Methods

4.4.1. Animals. Naïve adult male Sprague-Dawley rats (Charles River, Wilmington, MA) weighing 250-275 g at the beginning of the study were housed in individual cages and maintained on a 12:12-hr light-dark cycle (lights on at 0700) in a temperature and humidity-controlled vivarium. Animals were maintained on ad libitum water and standard laboratory chow, unless otherwise stated, and rats weighed 420-546 g at the start of experiments. All experiments were conducted in accordance with the Pennsylvania State Institutional Animal Care and Use Committee guidelines and approved protocols.

4.4.2. Test Solutions and Drugs.

Sucrose (Fisher-Scientific, Fair Lawn, NJ) was dissolved in filtered tap water (source identical to water available in home cages) and prepared no more than 24 hours prior to behavioral testing. For behavioral experiments, glucagon-like peptide 1 (7-36) amide (GLP-
1), Exendin-3 (9-39) amide (Ex-9), and angiotensin II were obtained from Tocris Biosciences (Ellisville, MO) and dissolved in sterile artificial cerebrospinal fluid (aCSF; Harvard Apparatus, Holliston, MA), which was also used as the control solution. Drugs were administered through a GENIE™ Infusion pump (Kent Scientific, Torrington, CT) at 1 µl/min for 2 min, for a total volume of 2 µl, with an additional 90 s to allow for diffusion prior to removal of the injector. Drugs were prepared differently for microiontophoresis and are detailed in below.

**4.4.3. Guide Cannula Surgeries for Lateral or Fourth Ventricle Infusions.**

Animals were anesthetized with ketamine/xylazine (90 mg/kg ketamine/9mg/kg xylazine). Using flat-skull procedures, rats were implanted stereotaxically with a guide cannula (22 gauge, Plastics One Inc, Roanoake VA) aimed directly above the fourth ventricle (AP: +2.5 mm from the occipital suture, ML: on the midline, DV: -7.0 mm) or the lateral ventricle (AP: -0.8 mm, ML: ±1.5 mm, DV: -3.5 mm), coordinates chosen from Paxinos and Watson (Paxinos and Watson, 2007). Guide cannula was secured to the skull with stainless steel screws and dental acrylic. Obturators (Plastics One Inc, Roanoake VA) were kept in the guide cannula at all times to prevent occlusion. Animals were given 1 week for recovery prior to training sessions. At the conclusion of all experiments, animals with lateral ventricle cannula were tested for patency and cannula placement using the previously defined criterion that rats consume more than 5ml of water in 60 mins (Kinzig et al., 2002). At the end of experiments, all groups (lateral, fourth ventricle, and VTA cannula) were overdosed on ketamine/xylazine, transcardially perfused with heparinized saline followed by 4% formalin. Brains were removed and sections obtained with a cryostat (at 40 µm) prior to staining with cresyl violet to verify cannula placement. Any animals that did have accurate placements were excluded from all analyses.
4.4.4. Intracerebroventricular Infusion and Testing Procedures.

Immediately prior to infusions and until the end of the test session, food but not water was removed from the animals’ home cage. Testing took place in the light phase (1100-1300 hrs). Animals were trained in 8 daily training sessions in which a 100 mL bottle containing a sucrose solution (0.3M) was attached to the front of the home cage with a stainless steel spout extending into the home cage and animals were allowed to drink *ad libitum*. Once stable daily baseline intakes were achieved, animals were habituated to the infusion procedure. On the first day, animals were given a sham injection in which a short injector was inserted into the cannula, but not the ventricle, with no volume administered. On the second day, animals received aCSF through a full-length injector extending 0.5 mm into the ventricle. After habituation, animals received counterbalanced infusions of aCSF (control solution; 2 µl) and various doses of the peptide GLP-1 (7-36) amide (0.6, 2, 6, or 14 µg in 2 µL vehicle) immediately prior to access to 0.3M sucrose. This concentration was chosen as it is readily consumed by rats and therefore, is commonly used in experiments investigating palatability-driven intake (Bonacchi et al., 2008; Sclafani et al., 1998).

4.4.5. Immunohistochemistry.

Four male adult rats were deeply anaesthetized with Nembutal and transcardially perfused with heparinized physiological saline solution followed by 4% paraformaldehyde buffered in phosphate buffer (PB, 0.1 M pH 7.4). After removal from the skull, brains were immersed in 20% sucrose diluted in the same fixative used for the perfusion for 4 hours at room temperature (RT) than in 20% sucrose diluted in PB overnight at 4 ºC. Sections containing the ventral tegmental area were cut with freezing microtome at 40 µm, and free-floating sections were collected in PB. After three washes in phosphate buffer saline (PBS, pH 7.4) for 10 minutes each, sections were incubated in a blocking solution containing 0.3% Triton
X-100 (Sigma) diluted in PBS and additionally 10% of normal serum for 1 hour. Then the primary antibodies were diluted in PBS containing 0.3% Triton X-100 (Sigma) and 1% of normal donkey-serum and sections were incubated overnight at RT. The following primary antibodies were used: polyclonal rabbit anti-GLP-1R (Santa Cruz, Santa Cruz, CA, 1:200), monoclonal mouse anti-tyrosine hydroxylase (TH, Immunostar, Hudson, WI 1:2000) and polyclonal goat anti-glutamic acid decarboxylase 65/67 (GAD, Santa Cruz, Santa Cruz, CA1:200) antibodies. Then sections were washed three times 10 minutes with PBS and incubated with the following secondary antibodies: donkey anti-mouse Alexa488, donkey anti-rabbit Alexa568, donkey anti-goat Alexa647 (Invitrogen, Carlsbad, CA). Nuclear counterstaining was performed with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, 1:50,000, Invitrogen, Carlsbad, CA). Sections then were washed in PBS mounted on gelatin-coated slides and covered with Fluoromont G (Southern Biotech, Birmingham, Alabama). Immunoreaction was examined under Olympus Fluoview V1000 confocal laser scanning microscope and acquired images were edited with the Adobe Photoshop7 software.

4.4.6. Evaluation of immunohistochemistry data

For semiquantitative evaluation, sections containing ventro-medial and dorso-lateral regions in different rostro-caudal extensions of the VTA were photographed with 40x objective and GLPR1- and TH-expressing cells and cells co-localizing GLP-1R and TH were separately counted. The percentage of GLP-1R-immunoreactive cells co-localizing TH and that of TH-immunoreactive cells co-localizing GLP-1R were calculated.

4.4.7. Electrophysiology Surgery and Preparation.

For anesthesia, isoflurane (Phoenix Pharmaceutical Inc., USA) was used, considering its possible effect on dopamine neurons, as an in vivo pharmacological experiments it is important to maintain a stable level of anesthesia to avoid potential effects on neuronal
activity. Furthermore, in a sub-group of rats (n=4) we tested the effect of Nembutal anesthesia (i.p.) to determine if we could reproduce all neuronal responses. The animal was placed in the anesthesia-induction chamber initially receiving 5% isoflurane on 800 ml/min oxygen flow. Once the animal was deeply anesthetized, it was transferred to a stereotaxic frame and placed on 2-3% isoflurane with 200 ml/min oxygen via nose cone to maintain a stable and easily adjustable anesthesia level with body temperature was maintained between 36-37°C using an adjustable heating pad (FHC Inc., USA). After the trepanation of the skull and the removal of the dura mater, a microiontophoretic electrode (Kation Scientific, USA) was lowered into the paranigral subdivision of VTA. The coordinates were: AP: -5.2 mm to -5.4 mm from bregma, ML: 0.6-1.0 mm, DV: 7.5 mm to 8.5 mm from the surface of the dura, according to the rat brain atlas of Paxionos and Watson (fourth edition, 1998).

4.4.8. Identification of Dopamine neurons.

In the present experiment, we aimed to record dopamine neurons in VTA, understanding and considering the inherent difficulties (Margolis et al., 2006). To identify dopamine neurons the following well accepted criterions were used: broad, frequently triphasic extracellular action potential with a duration more than 2 ms, relatively slow firing rate (<10 Hz), aversive stimuli (foot pinch) induced inhibition in neuronal activity was also observed (Ungless et al., 2004a). Additionally, as a positive control, we recorded from a subset of neurons while animals were under Nembutal anesthesia, and found neuronal responses to be indistinguishable from those observed while animals were maintained under isoflurane anesthesia (data not shown).

4.4.9. Neurochemical preparation and application.

Neurochemicals were juxtaneuronally delivered to dopamine neurons in the VTA using a microiontophoresis system (Neuro Phore BH-2, Harvard Apparatus) in combination with
extracellular single neuron recording (pre- and main amplifier, AM-system, USA; A/D converter Power micro 1401 with Spike 2, CED, UK) using six-barrel micropipettes with a tip-diameter of 10 µm (Kation Scientific, USA). The impedance of recording electrodes was 0.4-1.2 MΩ. The recorded data was sorted into single unit classes using off line waveform analysis (Spike 2 software, CED, UK). To ensure correct separation of single units overdrawn waveform comparisons and principal component analysis were performed. After the single units were separated, frequency histograms (spike/seconds) for each recorded neuron were made. GLP-1 (TOCRIS 2082, 300 µM/l dissolved in saline, 50-100 nA, `+` DC current for 30 sec) was administered to test the responsiveness of dopamine neurons in the VTA. Ex-9 (TOCRIS 2081, 300 µM/l dissolved in saline, 50-100 nA `−` DC current for 30 sec) was also applied alone or in combination with GLP-1 to test a) the effect of endogenous GLP-1, and b) whether the effects by GLP-1 was mediated by receptor specific mechanisms.

In order to examine expression of auto Dopamine D2 receptors (D2R), 38 pre-identified dopamine neurons were tested with Quinpirole (QP, D2R agonist, TOCRIS 1061, 39mM/l dissolved in distilled water, 100nA `+` DC current for 30 sec). Bicuculline methiodide, the selective post synaptic GABA_A receptor antagonist (TOCRIS 2503, 10 mM/l dissolved in distilled water, 100 nA `−/+` DC current for 30 sec) treatment was delivered 30 sec before and in parallel with GLP-1 application to test the possible GABA input dependent effect of GLP-1 on dopamine neurons. We used L-glutamic acid (Sigma-Aldrich G1626, 150 mM/l dissolved in distilled water, 50-100 nA `−` DC current for 10-30 sec) and γ-aminobutyric acid (GABA, Sigma-Aldrich A2129, 150 mM/l dissolved in 0.5M Na-acetate, 50-100 nA `+` DC current for 10-30 sec) for excitatory and inhibitory controls, respectively. One barrel filled with 2% Pontamine Sky Blue solution (Sigma-Aldrich C8679, dissolved in 0.5 M Na-acetate) served to deliver a balancing current to avoid electric stimulation of the recorded
neurons during microiontophoresis and also to label the recording site at the end of the session with an unbalanced ejection (500 nA, for 5-10 min). To avoid leaking or clogging, the impedance of the barrels were kept between 10-200 MΩ.

4.4.10. Statistical analysis.

Food intake (presented in kilocalories), body weight (presented in grams) and sucrose intake (presented as mL consumed and as percent reduction from aCSF baseline after GLP-1 infusion) were calculated as Mean ±SEM. Data was analyzed by a one-way analysis of variance (ANOVA) with GLP-1 dose (aCSF=0, 0.6, 2, 6, or 14 µg) as the independent factor. To evaluate the neural responses to the applied drugs during recording, the average firing rate (spikes/seconds ± SEM) over 30 sec before and after the onset of iontophoretic administration were compared by two-tailed paired samples t test (p<0.05). The analysis was applied for each iontophoretic application to decide whether the neuron responsive or not to neurochemicals. The assessment of individual neurons was used as the basal firing rate of dopamine neurons in the VTA is slow and therefore a little change in incoming neuronal control can double or half the basal activity resulting in a statistically non evaluable average neuronal activity. The neuron was considered to be modified by the neurochemical if the microiontophoresic application of that neurochemical caused significant alteration from the basal firing rate, and which could be repeated. In this way, categorized neurons were averaged (for basal firing rate and drug induced action potential rate) and subjected to two-tailed paired samples t test (p<0.05) again and further, separate ANOVAs were used to determine the various drug effects. All significant results (p < 0.05) were further analyzed using LSD post-hoc tests.
Chapter 5.

Central Glucagon-like peptide-1 plays a role in altered sucrose reward following Roux-en-Y gastric bypass in dietary obese rats

* This chapter includes results from the manuscript: Pritchett CE, Zharikov A, Rogers AM, Tomasko, JM, Popova E, Hajnal A. Central versus peripheral GLP-1 receptor modulation of operant sucrose licking in dietary obese rats following Roux-en-Y gastric bypass. Physiology and Behavior. Under Review.

5.1. Introduction

Obesity has become one of the leading health concerns in Westernized cultures (1992; Flegal et al., 2012; Ogden et al., 2012). Unfortunately, traditional treatments such as diet, exercise, and various weight loss drugs have met with a myriad of side effects and often lead to short-term results followed by rebound weight gain (Beckman et al., 2010; Vincent and Le Roux, 2008). Of particular concern is the rise in serious obesity-related endocrine and metabolic complications, such as type 2 diabetes mellitus (DM).

One emerging therapy, Roux-en-Y gastric bypass surgery (RYGB), has been shown to be particularly successful in achieving both weight loss and resolution of type 2 DM (Eldar et al., 2011). RYGB has traditionally been considered a combined restrictive-malabsorptive procedure which results in reduced appetite, decreased body weight and adiposity, improved glucose control, and changes in taste preferences such as a shift in sweet taste perceptions following the surgery (Hajnal et al., 2010; Le Roux et al., 2011; Shin and Berthoud, 2011; Shin et al., 2011). These changes cannot be fully explained by the loss of body weight alone, as they can occur prior to actual weight loss. This has led to the belief that early hormonal changes may be crucial factors in both initial weight loss and maintenance of lower body weight (Beckman et al., 2010; Hajnal et al., 2010; le Roux et al., 2006; Morinigo et al., 2006; Shin and Berthoud, 2011; Shin et al., 2011; Vincent and Le Roux, 2008). Despite these implications, the precise underlying mechanisms through which
RYBG may produce these changes in appetite, glucose improvement, hedonic valuation of food, and food choice; particularly in the phase immediately after surgery and prior to weight loss, remain to be elucidated (Shin and Berthoud, 2011; Vincent and Le Roux, 2008).

While data strongly suggest that obesity results in substantial changes to the reward system, it also has been shown to significantly influence the release of important pre-, and postprandial hormones such as ghrelin, Peptide YY, leptin and GLP-1, among others (Roberts et al., 2002; Verdich et al., 2001; Williams et al., 2011). Thus, one plausible mechanism through which RYGB may exert its effects could be through immediate changes in some of these gut-brain axis hormones. Of particular interest are the notable changes to release of the anorexigenic incretin hormone GLP-1 after the surgery (Morinigo et al., 2006; Pournaras et al., 2010a).

Both human patients and animal models have shown that GLP-1 levels are markedly increased before weight loss and immediately following RYGB (Beckman et al., 2010; le Roux et al., 2006; Morinigo et al., 2006; Pournaras et al., 2010a). In further support of the theory that GLP-1 may be involved in food reward is the recent data strongly linking GLP-1 to taste functions. GLP-1 receptors are co-expressed with markers for sweet taste signaling such as α-gustducin and T1R3, both in the intestine and on the tongue, where they may control the release of this and other hormones (Jang et al., 2007; Kokrashvili et al., 2009a; Kokrashvili et al., 2009b). Indeed, GLP-1 knockout mice exhibit altered taste sensitivity, and exogenous activation of GLP-1 receptors potently reduces the intake of sweet solutions in both hungry and sated animals (Alhadeff et al., 2012; Asarian et al., 1998; Martin et al., 2009; Pritchett and Hajnal, 2012; Shin et al., 2008).

Of further interest is the role of GLP-1 within the central nervous system. GLP-1Rs are localized in areas involved with food reward such as the nucleus accumbens, ventral
tegmental area, and various hypothalamic nuclei; these nuclei interact to mediate the intake of food based on its hedonic value, in part with relation to homeostatic need (Alvarez et al., 1996; Jin et al., 1988; Larsen et al., 1997a; Larsen et al., 1997b; Merchenthaler et al., 1999; Navarro et al., 1996; Thiele et al., 1997). Since the seminal paper by Turton and colleagues, activation of central GLP-1Rs has been shown to not only reduce homeostatically driven food intake, but also hedonic intake due to the palatable nature of the food (Dossat et al., 2011; Tang-Christensen et al., 1996; Tang-Christensen et al., 1998; Turton et al., 1996). Taken together, these data implicate a role for GLP-1 in the seeking and consumption of palatable foods, and this role may be highly influential in RYGB outcomes. In fact, recent studies have further supported central GLP-1 as being involved in food reward, with activation of GLP-1Rs in reward related areas such as the ventral tegmental area and the nucleus accumbens reduced intake of highly-palatable diets and treats (Alhadeff et al., 2012; Pritchett and Hajnal, 2012; Williams et al., 2011). Thus changes to central GLP-1 signaling following RYGB may be important for the long-term beneficial effects of the surgery, and perhaps could serve as a potential non-invasive treatment for obesity and its complications.

Despite these important implications, the extent to which GLP-1 engages the reward system, and particularly how it is affected by RYGB, remain unclear. Therefore, the current study investigated whether central or peripheral manipulations of GLP-1Rs are more efficient in altering incentive motivation for sucrose-seeking in dietary obese rats after RYGB. We hypothesized that dietary obese rats that had undergone RYGB surgery would be more responsive to exogenous GLP-1 than Sham controls, as dietary obesity appears to blunt the GLP-1 system while RYGB may restore that sensitivity. Furthermore, we expected that the central GLP-1 would be more effective at reducing sucrose reward seeking behaviors than GLP-1 administered peripherally.
5.2. Methods

5.2.1. Animals and diets

Male Sprague-Dawley rats weighing ~350 g at the beginning of the experiment (Charles River) were housed individually in stainless steel hanging cages in a temperature and humidity controlled vivarium on a 12:12 h light-dark cycle. After acclimation, rats were maintained on a high fat (HF) diet (Teklad Research Diets #D12492) consisting of 5.24 kcal/gram (60% kcal from fat, 20% kcal from carbohydrates and 20% kcal from protein) prior to surgery and throughout the experiment. HF diet and water was available ad libitum except when stated otherwise. Additional age-matched, surgery- and sucrose-naïve controls rats (n=5) were maintained on chow and water and sacrificed in an identical manner as RYGB and Sham rats to serve as an un-manipulated control group for qRT-PCR. All protocols and procedures were approved by The Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee.

5.2.2. Drugs

Both the GLP-1R agonist Exendin-4 (Ex-4) and the GLP-1R antagonist Exendin-3 (9-39) amide (Ex-9) were obtained from Tocris Biosciences (Ellisville, MO). For experiment 1, peripherally administered doses of Ex-4 (0.5, 1.0, 1.5, and 2.0 µg/kg; ip) and Ex-9 (10, 50, and 100 µg/kg; ip) were dissolved in sterile 0.9% saline, which also served as the vehicle solution. These doses were chosen based on previous studies (Hayes et al., 2011; Pritchett and Hajnal, 2012). For experiment 2, ICV administered Ex-4 (0.6, 1.0, 2.0 µg) and Ex-9 (20 µg) were dissolved in 2 µl sterile artificial cerebrospinal fluid (aCSF; Harvard Apparatus, Holliston, MA), which served as the vehicle solution for experiment 2. These doses were chosen from previous literature (Hayes et al., 2008; Hayes et al., 2009; Williams et al., 2009) and pilot studies in our laboratory, and were administered in counterbalanced order, with a
minimum of one control injection day between each drug injection day to ensure drug washout and stable baselines.

5.2.3. Roux-en-Y gastric bypass surgery

Animals were maintained on the high fat diet for 14 weeks prior to receiving either RYGB or Sham operation. A detailed description of the surgical technique and perioperative care for RYGB has been published previously, except that Sprague-Dawley rats were used in the current study (Hajnal et al., 2010b). Briefly, rats were fasted overnight, but allowed water, prior to the surgery. Anesthetized rats (isofluorane: 3% for induction, 1.5% for maintenance) were maintained under sterile conditions and were pretreated with a prophylactic antibiotic (Ceftriaxone: 100 mg/kg, im; Roche, Nutley, NJ). An abdominal incision was made on the midline and, in the RYGB surgeries, the stomach was divided to create a reduced (20%) gastric pouch using a blue load GIA stapler (ETS-Flex Ethicon Endo surgery, 45 mm). Measuring from the ligament of Trietz, the small intestine was divided to create a 15 cm biliopancreatic limb and a 15 cm alimentary “Roux” limb, with the remaining segment (65-70 cm) forming the common channel. The gastrojejunal and jejunoojejunostomies were performed end-to-side using interrupted 5-0 polypropylene sutures and the abdominal wall and skin were closed using 3-0 silk and 5-0 nylon. The Sham-operated controls received manipulation of the stomach and a transverse enterotomy at the same level of the proximal jejunum; this was reclosed with interrupted 5-0 polypropylene sutures without forming an anastomosis. The stomach was freed from caudal attachments, and a space along the lesser curve was cleared of attachments as if a stapler was to be inserted. However, the stomach was then replaced to its anatomic location without further manipulation.
To minimize postoperative pain, all surgical incisions were treated with 0.5 ml of subcutaneous 0.25% bupivacaine. Further postoperative care included treatment with normal saline (sc; 50 ml/kg, immediately prior to surgery and after surgery, and on postoperative day 1) and buprenorphine (0.5 mg/kg, im) as needed for pain. Animals were given 24 hrs for the anastomoses to heal before being allowed to eat or drink, at which point animals were maintained on a liquid diet consisting of BOOST® (Nestle Nutrition, Minneapolis, MN) and access to water ad lib for 3 days. On postoperative day 3, the Boost was removed and animals were returned to their high fat diet.

5.2.4. Lateral ventricle surgery and verification

Two months following the RYGB surgery, after they had completed training and the peripheral portion of the study, the animals were anesthetized with a ketamine-xylazine mixture (90 mg/kg+9 mg/kg) and placed in the stereotaxic surgery frame (Kopf Instruments, Tujunga, CA). Using standard aseptic surgical procedures, stainless steel indwelling guide cannula (5 mm length, 23 gauge; Plastics One, Roanoke, VA) were unilaterally implanted directly above the lateral ventricle, alternating between left and right sides (from Bregma: AP: -0.8 mm; ML: ±1.5 mm; DV: -3.5 mm; coordinates based off Paxinos and Watson (Paxinos and Watson, 2007)). The cannula was affixed to the skull using self-curing dental acrylic adhered to 3-4 stainless steel screws placed in different quadrants of the skull. Obturators (Plastics One, Roanoke, VA) were kept in the guide cannulae at all times to prevent occlusion.

At the end of all experiments, cannula placement was verified by using the common method of intracerebroventricular administration of angiotensin II (50 ng in 2 µl; Tocris Biosciences, Ellisville, MO). Water intake was monitored for 15 min, any animals that did not drink ≥ 5 ml were excluded from all analyses.
5.2.5 Analysis of body weight

To analyze body composition differences in RYGB and Sham rats, and to demonstrate the presence of obesity in Sham rats, $^1$H-NMR (Brucker LF90 proton-NMR Minispec, Brucker Optics, Woodlands, TX) body composition analysis was conducted between experiments 1 and 2 (prior to lateral ventricle cannulation and experiment 2), at approximately 3 months post-surgery.

5.2.6. RNA isolation and qRT-PCR

At the end of all experiments, non-food-deprived animals were anesthetized with ketamine/xylazine immediately prior to rapid decapitation. Brains were immediately harvested and flash-frozen for qRT-PCR. Tissues from naïve chow-fed non-surgical control rats were also harvested in an identical manner for qRT-PCR comparisons.

GLP-1R mRNA levels were determined by quantitative real-time PCR. Cyclophilin A mRNA levels were measured in each sample to serve as internal controls from which to normalize GLP-1R mRNA levels (Li et al., 2003; Zhou et al., 2003). Primers (cyclophilin A, Gene Symbol: PPIA, GenBank no. M15933 and GLP-1R: Gene Symbol: Glp1r, GenBank no NM_012728) were obtained from Qiagen and were specific for rat and use with SYBR® Green-based real-time RT-PCR (Cyclophilin, Cat. No: QT00177394; GLP-1R, Cat No: QT01084825).

Using the RNeasy mini kit (No. 74104, Qiagen, Valencia, CA), total RNA was isolated from the cortex and midbrain of RYGB, Sham and the naïve chow-fed Control rats, and from the liver and pancreas of Control rats to serve as the positive control tissue for GLP-1Rs. Total RNA from each sample was quantified spectrophotometrically and cDNA was synthesized from 200 ng/μl isolated RNA using the SuperScript III First-Strand Synthesis SuperMix (Invitrogen Corporation, Carlsbad, CA). qRT-PCR was performed using
an iCycler IQ™ Real time PCR detector system (Bio-Rad, Hercules, CA). The reaction was performed in a 96-well plate with the following reaction mixture (per 20 µl reaction) added to each well: 1X iQ™ SYBR Green SuperMix (Bio-Rad), 340 ng/µl cDNA, and primer according to kit instructions. Cyclophilin and GLP-1R primers obtained from Qiagen were prepared and used according to the manual. The thermal cycle condition was initialized at 95°C for 3 min for one cycle followed by 40 cycles at 95°C for 15 sec for denaturation, annealing at 55°C for 30 sec and extension at 72°C for 30 sec, followed by 72°C for 10 min.

5.2.7. Apparatus

Testing took place in one of six identical operant chambers (MED Associates, St. Albans, VT) in a room separate from the colony room. All chambers had clear Plexiglas tops, front and back walls. The chambers measured 30.5 x 24.0 x 29.0 cm (length x width x height) with a grid floor above a removable waste tray. Each chamber was equipped with three retractable sipper spouts that entered through 1.3 cm diameter holes placed 16.4 cm apart. Each chamber was equipped with a 25W house light within in a light and sound attenuated cubicle fitted with a white noise source (75 dB). Sucrose reinforcement was monitored by a lickometer circuit in which licks on an empty bottle (the active or operant spout) triggered deployment of a second bottle containing 0.3M sucrose for 10 seconds, during which time licks from the sucrose spout were recorded.

5.2.8. Training, PR schedule, and drug testing

Our standard progressive ratio (PR) training and testing procedures have been described in detail elsewhere (Hajnal et al., 2007a). Briefly, after recovery from RYGB surgery and animals had obtained stable body weights, rats were overnight water deprived for continuous access training. For four days, rats received 30 min access to water in the operant chambers, with additional 2 hr access to water each afternoon in their home cages to ensure
proper hydration. Following the 4 days of water training, rats were returned to ad libitum water access for the remainder of the testing period. Sucrose training then commenced, with rats given three consecutive days of testing on one of four ascending sucrose concentrations (0.03, 0.1, 0.3, and 1.0 M), with only one concentration tested per day. Once stabilized lick responses were obtained, training on a fixed ratio schedule (FR) began. Rats were placed in the operant chambers with three spouts: Spout 1 (left – “sucrose” spout) contained one of the four sucrose concentrations) while spouts 2 (middle – “active/operant” spout) and 3 (right – “inactive” spout) were empty. Upon program activation, empty spouts 2 and 3 were presented, with licks on the inactive spout producing no programmed consequences and licks on the active spout counting towards completion of the FR-10 lick contingency schedule. That is, 10 licks on the active spout produced retraction of the two empty spouts and 10-s presentation of the sucrose spout. At the end of the 10-s interval, the sucrose spout retracted and the procedure was repeated across a 30-min session. Once animals were trained on the FR 10 schedule (12 days), they were moved to the more rigorous progressive ratio-10 schedule (PR10), which was identical to the FR10 schedule, except that the requirement for sucrose access increased by 10 licks per reinforcement (PR10: i.e. -10, -20, -30, etc.). However, if animals did not meet the requirement after 10 minutes, the session was terminated without a reinforcement reward, providing the animals’ breakpoint (operationally defined as the number of reinforcement cycles completed). Licks on all three spouts and latency to lick the active spout and sucrose spout were also measured. Once stable licking on PR10 was identified (12 days), drug testing commenced.

Control vehicle or drugs were administered (ip or icv) 15 minutes prior to being placed in the operant chambers on a PR-10 schedule of reinforcement for 0.3M sucrose in counterbalanced order. Previous research from our laboratory has shown that Sham rats
maintained on a HF diet have increased preference for higher sucrose concentrations, and RYGB rats have increased preferences for lower sucrose concentrations (Hajnal et al., 2010; Shin et al., 2011; Tichansky et al., 2011). Thus, 0.3M sucrose was chosen as a moderate concentration that both groups readily consumed with the smallest difference in responding between groups during training. A minimum of 48 hrs was given between ip or icv drug injections. On between-drug days, rats were treated with the appropriate vehicle and tested under conditions identical to the drug days. Thus each dose had a preceding vehicle day to serve as a baseline.

All peripheral testing (Sham=6; GBS=5) took place prior to implantation of the lateral ventricle cannula. Additional age- and surgery-matched animals that had also completed training in the operant chambers were then included to increase total n-values (Total: Sham=9; GBS=7). Animals were given 12 days after peripheral testing for surgery and recovery and then all animals were given an additional 3 days on PR10 with no infusions. No significant differences in baseline responding were observed between the added animals and those that completed peripheral testing (experiment 1), therefore all animals in experiment 2 were analyzed together.

5.2.9. Statistical Analysis

Body weight and food intake were measured daily and the change across 24 hrs was also calculated. Data from progressive ratio sessions were averaged within groups and are presented as Mean ± SEM. Factorial ANOVAs were conducted with Group (Sham or RYGB) and Treatment (ip: Ex-4, 0, 0.5, 1.0, 1.5, 2.0 µg/kg and Ex-9, 0, 10, 50, 100 µg/kg or icv: Ex-4, 0, 0.6, 1.0, 2.0 µg and Ex-9, 0, 20 µg) as the independent factors and 24 hr food intake, breakpoint, and spout licks (active, inactive and sucrose reward) as the dependent measures. Daily body weight and NMR data was analyzed using one-way ANOVA with
Group (RYGB or Sham) as the independent factor. All significant effects were further analyzed using Fisher LSD post-hoc tests. GLP-1R mRNA expression was measured using qRT-PCR and results are normalized to cyclophilin A mRNA. The data were analyzed using two-way ANOVA with Group (RYGB or Sham) and tissue site (Cortex or Midbrain) as the independent factors with data shown as Mean ± SEM. Data were analyzed using Statistica 7.0 (StatSoft, Tulsa, OK).

5.3. Results

5.3.1. Body weight and food intake results

Preoperative body mass for RYGB and Sham was 834 ± 26 g, and 895 ± 77 g, respectively, and statistically not different. Whereas all rats lost weight uniformly by the end of the third post op week, by the end of the 4th postoperative week RYGB rats displayed a loss of ~22% to their presurgical weight (657 ± 20 g) compared to the sham-operated rats (p < 0.05) who had almost completely regained body weight to the preoperative level (834 ± 35 g). Three months after RYGB surgery and after recovery from the lateral ventricle cannula surgery, at the beginning of testing the body weight of the RYGB rats had returned to a steady level of ~85%, whereas Sham rats had gained ~10% more than their initial preoperative weight. Throughout the experiment, RYGB and Sham rats differed significantly in body weight (average daily weight; RYGB: 668 ± 4.5 g; Sham: 924 ± 8.3 g). The one-way ANOVA revealed a significant effect of surgical condition (F(1,476)=617.245, p < 0.001), with RYGB rats weighing significantly less throughout the experiment, despite continued consumption of the high fat diet. Figure 5.1a shows the body weight differences of RYGB and Sham rats throughout the experiment (p < 0.001; all time points).
Figure 5.1. Changes in body composition following recovery from Roux-en-Y surgery. Roux-en-Y gastric bypass surgery produced significant changes in body weight and composition measured approx. 3 months after the surgical procedures. Despite maintenance on a high-fat diet, RYGB produced significant reductions in overall body weight (a) and in the percentage of body weight attributable to fat mass and an increased percentage of lean muscle mass (b). Sham compared to RYGB, * p < 0.05; *** p < 0.001.
Figure 5.1b depicts the difference in RYGB and Sham rats body composition measured via $^1$H-NMR at a halfway point in the study (between Experiments 1 and 2, i.e. postoperative 3 months). ANOVA revealed a statistically significant difference in the percentage of lean mass ($F(1,13)=5.084$, $p < 0.05$) and fat mass ($F(1,13)=6.411$, $p < 0.05$) in RYGB animals compared to Sham.

Food intake was measured and calculated during the 24 hours after peripheral saline or GLP-1R agonist and antagonists treatments for both groups. The ANOVA revealed a significant effect of Group ($F(1,72)=244.446$, $p < 0.001$), Treatment ($F(7,72)=2.678$, $p < 0.05$) and a Group x Treatment interaction ($F(7,72)=2.146$, $p < 0.05$). Post-hoc tests revealed that RYGB rats consumed more of the HF diet than Sham rats at all doses (all: $p < 0.001$) and that intake was reduced in RYGB, but not Sham rats, following all but the lowest dose of Ex-4, which reduced intake by 85% following saline (0.5 µg/kg: $p=0.06$; 80% of baseline, 1 µg/kg: $p < 0.01$; 79% of baseline, 70% of baseline 1.5 µg/kg: $p < 0.01$; 2 µg/kg: $p < 0.001$). Ex-9 increased intake following the highest dose tested to 110% of baseline intake (100 µg/kg: $p < 0.05$).

The average amount of high fat diet consumed 24 hours after central treatment with aCSF, Ex-4 or Ex-9 is shown in Figure 5.2. ANOVA revealed a significant effect of Treatment ($F(5,83)=4.7967$, $p < 0.001$) but not Group ($F(1,83)=1.780$, $p=0.186$) or a Group x Treatment interaction ($F(5,83)=1.288$, $p=0.277$). Post-hoc tests revealed a significant reduction in 24 hour HF diet intake following 2.0 µg Ex-4 treatment in Sham and RYGB rats ($p < 0.05$), and this reduction was blocked by pre-treatment with 20 µg Ex-9 ($p=0.10$ compared to aCSF baseline).
Figure 5.2. Changes in food intake throughout Experiment 2 during central treatments. As in Experiment 1, food intake was measured daily, averaged across groups and compared as a function of treatment and surgical condition. As a trend throughout experiment 2, RYGB rats consumed more high fat diet daily than Sham rats, but this was not significant. There was a reduction in overnight food intake following the highest dose tested (2 μg Exendin-4). Sham compared to RYGB, *** p < 0.001.

5.3.2. Experiment 1: stimulation and antagonism of peripheral GLP-1 receptors

Figure 5.3 depicts the breakpoint that Sham and RYGB rats reached when working for 0.3M sucrose reinforcements on a progressive ratio-10 (PR10) schedule following saline or peripheral Ex-4 treatment. Table 5.2 details the number of licks made on the active, inactive and sucrose spouts. We found that peripheral administration of Ex-4 was not sufficient to decrease PR10 responding for sucrose. ANOVA revealed a significant
difference between Sham and RYGB rats ($F(4,69)=22.165$, $p<0.001$) but not Treatment ($F(28,250)=0.9838$, $p=0.493$) or a Group x Treatment interaction ($F(28,250)=0.9021$, $p=0.612$). Post-hoc analysis revealed that on several days RYGB rats had significantly higher breakpoints than Sham rats ($p<0.001$; Figure 5.3) and made significantly more licks on the active ($p<0.001$) and sucrose ($p<0.001$) spouts, as well as inactive licks ($p < 0.05$). Such changes in sucrose taste following RYGB have been previously demonstrated before and thus these differences were not unexpected (Hajnal et al., 2010; Shin et al., 2011; Tichansky et al., 2011). However, the peripheral Ex-4 treatments did not remarkably alter the behavior of either group working for 0.3M sucrose.

![Graph showing break point to work for a sucrose reward is unaffected by peripheral treatments.](image)

**Figure 5.3.** The breakpoint to work for a sucrose reward is unaffected by peripheral treatments. RYGB and Sham rats were tested on a PR-10 schedule of reinforcement and the number of completed cycles (breakpoints) were averaged and compared. In general, RYGB rats completed slightly to significantly more cycles, resulting in higher average breakpoints. Peripheral treatments of Exendin-4 or Exendin-9 prior to testing did not produce any significant trends in the behavioral responses of either RYGB or Sham rats. Sham compared to RYGB, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. 

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Ex-9 administered peripherally also did not significantly alter responding (Table 5.2). ANOVA showed significant differences between Groups \((F(4,51)=9.1349, p<0.001)\) but no effect of Treatment \((F(20,170)=0.7856, p=0.728)\) or a Group x Treatment interaction \((F(20,170)=0.6169, p=0.897)\). Post-hoc tests again revealed differences between RYGB and Sham rats in breakpoints \((p<0.001)\), active \((p<0.001)\) and inactive \((p < 0.05)\) spout licks and sucrose spout licks \((p<0.01)\), while Ex-9 treatment did not alter these behaviors. The latency to lick the active and sucrose spouts were also analyzed, with no significant effect of group, treatment or an interaction on latency to lick (data not shown).

5.3.3. Experiment 2: stimulation and antagonism of central GLP-1 receptors

When applied centrally, Ex-4 produced significant changes in the motivation to procure a sucrose reward and these changes were blocked by pre-treatment with the GLP-1R antagonist Ex-9 (Figures 5.4 and 5.5, Table 5.2). ANOVA revealed a significant effect of group \((F(4,212)=6.9422, p<0.001)\) and treatment \((F(60,829)=1.6988, p<0.001)\), but not a group X treatment interaction \((F(60,829)=0.7637, p=0.906)\). Post-hoc tests revealed RYGB and Sham rats differed significantly in breakpoint \((p<0.001; \text{Figure 5.4})\), active spout licks \((p<0.001; \text{Table 5.2})\) and sucrose spout licks \((p<0.0001; \text{Figure 5.5})\). We found that 2 µg Ex-4 significantly reduced breakpoint in Sham \((p<0.05)\) and RYGB \((p<0.05)\) rats and reduced the number of licks on the sucrose spout \((\text{Figure 5.5}; \text{Sham: } p<0.05; \text{RYGB: } p<0.05)\) but did not change the number of inactive spout licks \((\text{Table 5.2}; \text{Sham: } p=0.77; \text{RYGB: } p=0.63)\). Post-hoc tests also revealed that pretreatment with central Ex-9 blocked the reduction in breakpoint \((\text{compared to 2 µg Ex-4}; \text{Sham: } p<0.05; \text{RYGB: } p<0.05)\) and sucrose spout licks \((\text{Sham: } p<0.05; \text{RYGB: } p<0.05)\), without producing significant changes when administered alone on cycles \((\text{Sham: } p=0.633; \text{RYGB: } p=0.50)\), sucrose licks \((\text{Sham: } p=0.64; \text{RYGB: } p=0.46)\), or inactive spout licks \((\text{Sham: } p=0.87; \text{RYGB: } p= 0.44; \text{Table 5.2})\).
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<th>Saline (ip)</th>
<th>Exendin-4 (ip)</th>
<th>Exendin-9 (ip)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>0.5 µg/kg</td>
<td>1.0 µg/kg</td>
</tr>
<tr>
<td><strong>Active Licks</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>171 ± 62</td>
<td>152 ± 56</td>
<td>238 ± 49</td>
</tr>
<tr>
<td>RYGB</td>
<td>549 ± 145</td>
<td>571 ± 118</td>
<td>508 ± 129</td>
</tr>
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<td><strong>Sucrose Licks</strong></td>
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<tr>
<td>Sham</td>
<td>324 ± 84</td>
<td>307 ± 58</td>
<td>378 ± 50</td>
</tr>
<tr>
<td>RYGB</td>
<td>605 ± 102</td>
<td>603 ± 100</td>
<td>545 ± 116</td>
</tr>
<tr>
<td><strong>Inactive Licks</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Sham</td>
<td>5.7 ± 1.7</td>
<td>13.2 ± 3.9</td>
<td>33 ± 13.0</td>
</tr>
<tr>
<td>RYGB</td>
<td>27.3 ± 6.5</td>
<td>37.0 ± 14.8</td>
<td>20.0 ± 7.7</td>
</tr>
<tr>
<td><strong>Group Sig</strong></td>
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Table 5.1. Responses during progressive ratio sessions 15 minutes after peripheral treatments (Mean ± SEM). * p < 0.05, *** p < 0.001.
Figure 5.4. RYGB and Sham animals’ breakpoint to work for a sucrose reward is altered by central treatments. While on a PR-10 schedule of reinforcement, RYGB and Sham rats were tested following central infusion (lateral ventricle, icv) of Exendin-4 or Exendin-9. We found that 2 μg Exendin-4 produced significant reductions in the breakpoint for both RYGB and Sham rats working for a sucrose reward. This effect was blocked by pretreatment with the GLP-1 receptor antagonist Exendin-9. Compared to aCSF baseline # p < 0.05; 2 μg Exendin-4 alone compared to 2 μg Exendin-4 + 20 μg Exendin-9, * p < 0.05.
Figure 5.5. Intake of the sucrose reward is altered by central treatments in RYGB and Sham rats. During the PR-10 sessions, the number of licks made on the reward spout containing 0.3M sucrose was recorded. Following the highest Exendin-4 dose (2 μg), both Sham and RYGB groups reduced the number of licks for the sucrose reward. The reduction was blocked by Exendin-9 treatment. Compared to aCSF baseline # p < 0.05; 2 μg Exendin-4 + aCSF compared to 2 μg Exendin-4 + 20 μg Exendin-9, * p < 0.05.

Neither Ex-4 nor Ex-9 significantly altered the latency to lick from the active or sucrose spouts, as seen by ANOVA (group: F(2, 212) = 2.060, p = 0.130; treatment F(30, 424) = 0.747, p = 0.833; group X treatment: F(30, 424) = 0.810, p = 0.754; data not shown).


<table>
<thead>
<tr>
<th></th>
<th>aCSF (icv)</th>
<th>Exendin-4 (icv)</th>
<th>Exendin-9 (icv)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.6 µg</td>
<td>1.0 µg</td>
<td>2.0 µg</td>
</tr>
<tr>
<td><strong>Active Licks</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Sham</td>
<td>176.2 ± 39.1</td>
<td>199.8 ± 40.6</td>
<td>203.2 ± 55.6</td>
</tr>
<tr>
<td>RYGB</td>
<td>338 ± 138.5</td>
<td>340.6 ± 138.5</td>
<td>393.0 ± 146.2</td>
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<tr>
<td><strong>Inactive Licks</strong></td>
<td></td>
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<tr>
<td>Sham</td>
<td>7.4 ± 2.7</td>
<td>2.4 ± 1.1</td>
<td>5.2 ± 1.6</td>
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<tr>
<td>RYGB</td>
<td>10.8 ± 2.4</td>
<td>7.1 ± 1.7</td>
<td>15.3 ± 3.3</td>
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</table>

Table 5.2. Responses during progressive ratio sessions immediately after central treatments (Mean ± SEM). * p < 0.05.

5.3.4. Analysis of brain GLP-1 receptors using RT-PCR

Following behavioral testing, the midbrain region of RYGB, Sham and naïve Chow-fed control rats was extracted, and Figure 5.6 illustrates the qRT-PCR products generated from the extracted RNA from both the midbrain and a negative control sample, the cortex. GLP-1Rs were measured relative to the expression of cyclophilin A mRNA, which did not significantly differ between the three groups. ANOVA found a significant effect between cortex and midbrain samples (F(1,30)=1.6988, p < 0.001) but not Group (F(2,30)=1.6988, p=0.09) or a Group by Tissue interaction (F(2,30)=2.915, p=0.07). Despite the lack of statistical significance, midbrain GLP-1R expression appears to be greater following RYGB surgery compared to Sham animals (Figure 5.6). As previously shown in naïve control animals, in all groups there was little to no expression of GLP-1R mRNA in the negative control samples of the cortex (Dunphy et al., 1998; Goke et al., 1995).
RYGB rats showed increased GLP-1 receptors in the midbrain. Testing for GLP-1 receptor mRNA in the midbrain of RYGB and Sham rats using qRT-PCR indicated an increase in mRNA for the GLP-1 receptor in the midbrain region of the central nervous system following RYGB surgery compared to Sham but not naïve controls. Cortex samples served as a negative control, GLP-1 receptor mRNA shown as a ratio to cyclophilin A mRNA.

5.4. Discussion

The frequency with which gastric bypass surgery is being used as a mode of obesity and metabolic treatment in patients has risen in the past decade, calling for further investigation of the changes associated with this surgery (DeMaria et al., 2002; Eldar et al., 2011; Karlsson et al., 2007). Thus in the present study, we examined how dietary obese rats responded for a sucrose reinforcement following RYGB or Sham surgery in the presence or absence of GLP-1R activation or blockade. We found that central, but not peripheral activation of GLP-1Rs was sufficient to reduce responding for a palatable sucrose solution presented as a treat (i.e. in addition to ad libitum access to the high fat maintenance diet) in
both RYGB and Sham animals. Interestingly, peripheral treatments to the GLP-1Rs that were ineffective at altering sucrose responding did produce significant changes in overnight food intake in RYGB but not Sham rats. This finding suggests that homeostatic and hedonic eating may be differentially regulated by GLP1Rs, and is consistent with other findings that obese rats have a blunted sensitivity to peripheral GLP-1, which may have led to the lack of an effect in reducing food intake in our study (Pritchett and Hajnal, 2012). We also observed that when the GLP-1 receptor specific antagonist, Exendin-9 was administered, it blocked changes observed by GLP-1R activation, indicating the effects were specific to the GLP-1Rs. Overall, this data indicates that while both central and peripheral GLP-1 systems are involved in general food intake, central GLP-1 may be more involved in food intake related to the rewarding value of the food than the peripheral GLP-1 system. Furthermore, we found that central GLP-1R mRNA expression was altered following RYGB, representing a novel change in the neural system brought about by this surgery.

The lack of an effect in the peripheral studies could be the result of one of several factors. For example, it could be due to the doses simply not being sufficient to alter sucrose responses. Interestingly, a recent study in lean rats found that doses of Ex-4 within the range tested in the present study were able to reduce responding for sucrose pellets (Dickson et al., 2012). Previous research has indicated that the GLP-1 system is blunted in dietary-induced obesity (Pritchett and Hajnal, 2012; Williams et al., 2011), suggesting our rats, particularly the Sham rats, may have required higher doses. However, the same doses of Ex-4 that did not alter conditioned responses did reduce unconditioned, overnight food intake, while Ex-9 increased overnight food intake. Therefore, these doses were at least sufficient to alter other general intake behaviors over a 24-hour period and thus other possibilities must be explored. Another explanation may be whether or not the concentration of sucrose was appropriate to
see differences. As we used the same concentration (0.3M sucrose) for the central studies in which an effect was observed, the concentration of the test solution was likely sufficient to see differences in the peripheral study as well. This concentration was chosen from the range of sucrose concentrations used in training, as it was a mid-range concentration that both groups responded to at the most similar rates. Previous literature has shown that obesity shifts the sucrose-taste concentration curve to the right, while RYGB surgery can reverse this effect (Hajnal et al., 2010; Shin et al., 2011; Tichansky et al., 2011), although one recent report found no effect of RYGB during sucrose brief-access testing (Mathes et al., 2012). An alternative explanation is that signals conveyed from peripheral GLP-1Rs, in contrast to central GLP-1Rs, are not critical to the assignment of incentive value and/or conditioned responses for food reward. Other recent investigations strongly support the belief that central GLP-1Rs may be both necessary and sufficient to influence food reward-related behaviors (Alhadeff et al., 2012; Dickson et al., 2012).

Based on these findings, in the final part of the study we looked at the levels of GLP-1R expression in the midbrain, which contains reward-related areas such as the ventral tegmental area. We chose this region as recent studies have indicated a strong possible role for GLP-1Rs within these nuclei to influence food reward (Alhadeff et al., 2012; Dickson et al., 2012; Goke et al., 1995; Merchenthaler et al., 1999). Thus we measured levels of GLP-1R mRNA expression in the midbrain and cortex and compared Sham and RYGB groups to a naïve, chow-fed control cohort. As previous studies have shown, we found little to no expression in our cortex samples, but higher levels of expression in the midbrain (Goke et al., 1995; Li et al., 2003; Merchenthaler et al., 1999). Of great interest was the difference in GLP-1R expression in RYGB rats compared to Sham; we found that RYGB rats had a trend for higher levels of GLP-1R expression compared to Sham (obese) animals (Figure 5.6).
While these data indicate that GLP-1Rs within the brain of our DIO rats may return to baseline following RYGB surgery, further studies are needed to determine the time course over which central GLP-1Rs may return to the levels seen in controls following RYGB.

Previous literature has looked at a number of gut-brain hormones following RYGB and one must consider the possibility that other hormones, which are also involved in food reward modulation, may be altered by obesity and RYGB. Also altered following RYGB are hormones such as ghrelin and leptin (Pournaras et al., 2010b; Strader and Woods, 2005). Reviews of the various weight loss surgical procedures indicate that like GLP-1, other anorexigenic hormones such as postprandial PYY have been shown to increase following RYGB, along with oxyntomodulin, and possibly GIP and the pancreatic peptides, while RYGB appears, at least in the short term, to lower the orexigenic hormone ghrelin; other gastrointestinal hormone functions appear to be unaltered by RYGB, such as cholecystokinin (CCK) and basal PYY (Beckman et al., 2010; Vincent and Le Roux, 2008). Changes in leptin, which has been shown to interact with the GLP-1 system, are less consistent but several studies do indicate significant and possibly impactful changes in leptin; like GLP-1, leptin may increase following RYGB prior to significant weight loss (Beckman et al., 2010). Although the roles of these hormones are likely important in various degrees to the outcomes of RYGB, this data is among the first to report how one of these hormones, GLP-1, may be particularly involved in food reward and the motivation to consume high calorie palatable foods. Further research into GLP-1, alone and perhaps in combination with other hormones changed by RYGB, should lead to a greater understanding of how this surgical procedure can be a long-lasting and effective treatment for obesity.

Indeed, in light of the rising prevalence of obesity and the success of bariatric surgery compared to traditional weight loss treatments, research on the underlying mechanisms of
these operations could lead to non-surgical alternatives with comparable results. Understanding how bariatric surgery produces the changes in behavior that lead to sustained weight loss may be the best resource we currently have for future obesity treatments and prevention. We found that RYGB rats were more responsive to GLP-1R treatments in the peripheral portion of the study for unconditioned intake behaviors whereas the GLP-1R agonist, Ex-4 given directly into the brain, reduced conditioned responses for a sucrose reward in both Sham and RYGB rats, and these effects seem to be specific to the GLP-1R. We also show for the first time that dietary-induced obesity and RYGB surgery may have opposite but significant influences on the expression of GLP-1Rs in the brain. These results shed further light on the role of GLP-1 in RYGB and strongly indicate that the central GLP-1 system may be involved in food-reward related behaviors. Further research is needed, but GLP-1 appears be an important potential target for anti-obesity treatments.
Chapter 6.

Discussion

SUMMARY

Within the reviewed experiments, we investigated the impact of GLP-1 on hedonic intake and food reward behaviors in lean and dietary induced obese (DIO) rats fed one of two obesogenic diets. We compared peripheral GLP-1 stimulation responses to sucrose and fructose to responses following dopamine receptor inhibition. Chapters 2 and 3 demonstrate that the two DIO groups responded with significant differences, despite being of similar body weight. While all groups responded to GLP-1 stimulation and dopamine blockade with reductions in sucrose intake, we found responses varied depending on the test solution, sucrose or fructose, and the type of pharmacological manipulation that was used. As shown in Chapter 2, high fat (HFHE)-fed rats were more responsive than rats fed a fat-carbohydrate combination diet (FCHE) to both D1 and D2 receptor antagonists, except during the fructose test for D2 receptor antagonism. Interestingly, however, lean Chow-fed rats also did not respond to D2 receptor antagonism during the fructose challenge, but FCHE rats did, indicating that previous dietary history can have a significant impact on the dopamine system and later behavioral responses.

In Chapter 3, we measured sucrose and fructose intake a similar cohort of DIO and lean controls following peripheral administration of the GLP-1 receptor agonist Ex-4. Again, despite similar body weights, this investigation revealed that HF-fed rats were particularly unresponsive to GLP-1R stimulation, indicating a very high fat diet may be especially disruptive to the GLP-1 system and may lead to increased consumption of highly palatable sweet foods.
We next extended this investigation to the CNS in Chapter 4, using lean rats and measuring sucrose intake and conditioned responses for a sucrose reward following GLP-1R stimulation. We found that GLP-1 was ineffective at reducing these behaviors when administered in the lateral ventricle, but was highly effective when administered into the fourth ventricle, indicating the hindbrain may be more involved in such non-homeostatic feeding behaviors than the forebrain. We next localized the effects of GLP-1 to the VTA using pharmacological testing, demonstrating intra-VTA GLP-1 reduces sucrose intake and the motivation to procure and consume a sucrose reward in sated rats. Our results from immunohistochemistry clearly indicate that GLP-1Rs can be found on DA and GABA neurons within the VTA. Furthermore, application of GLP-1 directly onto VTA DA neurons demonstrated that GLP-1 functionally interacts with neurons in the VTA to modulate the neuronal firing rates of these neurons.

Finally, in Chapter 5, we measured if the willingness to work for a food reward may be different in DIO rats that had undergone RYGB versus a Sham surgery, and how the surgical procedure may influence the animal’s responses to central GLP-1R stimulation. This investigation revealed that RYGB animals would work slightly harder for a sucrose reward, and that although peripherally applied Ex-4 did not reduce the motivation of either group to work for a sucrose reward, central application of Ex-4 did have an effect. Interestingly, while both groups responded to central Ex-4 in both the sucrose tests and in overnight food intake, peripheral Ex-4 only produced a reduction in overnight food intake. Furthermore, GLP-1 receptor expression appeared to be slightly lower in DIO Sham rats while RYGB exhibited greater levels of GLP-1 receptors. These results indicate that RYGB rats may experience a change in central receptors related to feeding, and that sucrose reward related behaviros
appear to be responsive to central but not peripheral GLP-1R stimulation in HF fed rats following RYGB or Sham surgery.

Overall, the aim of these works was to establish the role of GLP-1 in the control of hedonic intake. Through the use of various behavioral, electrophysiological and neurochemical measures and pharmacological manipulations, we found that GLP-1 does indeed play a significant role in controlling palatable carbohydrate intake, that hindbrain sites may be more involved than forebrain areas for GLP-1 in hedonic sucrose intake and sucrose reward, that obesity can influence these effects, and that GLP-1 may be exerting its control via modulation of the mesolimbic dopamine system.

**SUGARS: THE PROBLEM OF HEDONIC EATING, FOOD REWARD AND OBESITY**

Sugar is a substance with a sweet taste and high energy content that humans, and many other organisms, are evolutionarily wired to find pleasurable and seek out for consumption (Ackroff, 2008; Sclafani, 1987). Not surprisingly then, sucrose and other sweets have been shown to activate nuclei along the mesolimbic dopamine pathway, and cause rats to engage in seeking behaviors similar to drugs of abuse, thus becoming known as a potent natural reward (Avena et al., 2012; Levine et al., 2003a). In rats, sucrose is a highly preferred carbohydrate, which rats exhibit preferences for over water and other sweeteners such as glucose and fructose, or artificial sweeteners (Ackroff and Sclafani, 2011; Sclafani and Abrams, 1986; Sclafani, 1987; Sclafani et al., 1998). Indeed, rats will engage in behaviors to seek out sucrose and will work for a sucrose reward (Avena et al., 2008; Sclafani, 1987).

These types of behaviors appear to be occurring with concurrent changes to the brain, particularly within the brain’s primary reward pathways; the mesolimbic dopamine and opioid systems, even leading to cross-sensitization between sucrose and drugs of abuse.
Studies in rats have shown that sucrose engages the dopamine system in a manner similar to certain drugs of abuse; increasing DA release, reducing binding to DA receptors, and upregulating the DA transporter (Avena et al., 2006; Bello et al., 2002; Bello et al., 2003; Colantuoni et al., 2001; Hajnal et al., 2004; Rada et al., 2005; Spangler et al., 2004). Of further interest is how potently sucrose engages and appears to alter the opioid system along the central reward pathway, much like in opioid abuse (Avena et al., 2012; Levine et al., 2003a). As seen during opiate abuse, animals fed sucrose have altered opioid mRNA, exhibit signs of opioid withdrawal when given an opioid receptor antagonist, altered brain activity when given an opioid antagonist, and opioid antagonists can block the sucrose intake (Avena et al., 2008; Colantuoni et al., 2001; Colantuoni et al., 2002; Pomoni et al., 2000; Spangler et al., 2004). Human studies have also revealed that sweet tastes activate limbic brain regions (Gearhardt et al., 2011).

In this way sucrose naturally engages the reward system to stimulate eating due to its palatability (hedonic eating), and leads to changes in the brain’s reward system. While the precise role of sucrose and other sugars in the human obesity problem is still questioned and studied, there is little doubt from animal research that significant changes occur during obesity that may influence sucrose intake, and that sucrose intake does have a significant and detrimental effect on body weight, and adiposity (Ackroff and Sclafani, 1988; Hajnal et al., 2005; Hajnal et al., 2007a; Hajnal et al., 2007b; Hajnal et al., 2009; Kanarek and Marks-Kaufman, 1979; Parnell et al., 2008; Ramirez, 1987; Reedy and Krebs-Smith, 2010; Roberts et al., 2002; White, 2008). As we learn more about the effects of obesity, it has become clear the problem may be multi-generational, as the offspring of obese rats show significant and obesogenic differences in taste preferences for palatable foods, behaviors of food reward and
motivation, and differences in the mesolimbic dopamine system (Levin, 2009; Naef et al., 2010; Ong and Muhlhausler, 2011).

Despite the debate of how sucrose has influenced obesity in the human literature, the animal literature leaves little doubt that understanding the control of sucrose intake, particularly with regard to obese states, is necessary with the current obesity epidemic. This is especially important with the rise in obesity among children and the plentiful supply of sugars in the diet (O'Reilly and Reynolds, 2012; Oken, 2009). Thus the current investigations studied the gut-peptide GLP-1, and how it may regulate sucrose intake.

CENTRAL GLP-1 AS A MODULATOR OF DOPAMINE AND HEDONIC INTAKE

Hedonic eating can have an adverse impact on food choices and meal size, and in turn, on body weight and metabolic regulation. Therefore, it is critically important to explore and determine the psychophysiological underpinnings of palatable food consumption, such as snacking on caloricly-dense but non-nutritive foods, and how such intake fits in with the cycles of overeating as a potential antecedent of obesity (Berridge et al., 2010; Volkow et al., 2011). Considering reports that an excess of calories in the U.S. diet are now consumed outside of homeostatic need in the form of highly-palatable, commonly high-fat and/or high-sugar snacks and beverages, it is crucial to understand how various neural and hormonal signals, such as GLP-1, may be involved (Archer et al., 2007; Briefel and Johnson, 2004). As such, the preceding studies were designed to understand how peripheral and central GLP-1 signaling plays a role in regulating the intake of a highly palatable treat based not on hunger or nutritive need, but more on the positive hedonic value of the food. We used sucrose, a common sweet carbohydrate that animals and humans find pleasurable and will seek to consume. We then determined that diet-induced obesity leads to a less responsive dopamine
and GLP-1 system and discovered that the type of carbohydrate ingested and dietary history interact in a complex manner. However, these findings led us realize that the GLP-1 system not only mediates non-homeostatic driven intake of sweet carbohydrates, but that it is differentially altered by the type of obesogenic diet. Furthermore, the findings of these studies closely mimic the findings of our dopamine system study, indicating both systems are influenced by dietary history and are altered in obesity.

Accordingly, the next step was to investigate the central effects of GLP-1 on hedonic intake of sucrose, and in sucrose reward. In Chapter 4 we localized the effects to the hindbrain and the ventral tegmental area (VTA). We are the first to report that GLP-1 receptors are indeed located upon dopamine neurons within the VTA, and that firing rates of these neurons can be directly and indirectly modulated by application of GLP-1. These findings are supported by reductions in both sucrose intake and the willingness to work for a sucrose reward, when GLP-1 is applied to the 4th ventricle or directly into the VTA. The behavioral findings were supported by finding co-expression of GLP-1 with DA markers in the VTA, and the ability of GLP-1 to alter firing rates by direct stimulation of dopaminergic VTA neurons.

Other recent studies support the findings that GLP-1 is indeed a modulator of food reward, and that it exerts these actions through the mesolimbic dopamine pathway. Alhadeff and colleagues recently provided some of the strongest support of the data described here, showing that the GLP-1 producing neurons of the NTS send projections directly to the VTA and the NAcc (Alhadeff et al., 2012). Furthermore, this study shows that food-deprived rats reduce their intake of both regular chow, a HF diet, or sucrose following GLP-1R stimulation with Ex-4 in either the VTA and NAcc, while Ex-9 increased HF diet intake when administered into the VTA or NAcc core (Alhadeff et al., 2012). In another recent report,
peripheral Ex-4 lowered the willingness to work for a sucrose reward, and suppressed the expression of a conditioned place preference for food (Dickson et al., 2012). Applied centrally, Ex-4 again reduced food motivation in an operant paradigm similar to those used within this report but using sucrose pellets. Similar to the studies described here with GLP-1, the investigators found that Ex-4 administered into the VTA and the NAcc shell reduced the motivation to work for and consume sucrose reward (Dickson et al., 2012). Furthermore, tests in sated and food deprived rats demonstrated that food reward behaviors are mediated by GLP-1R activation in the VTA by direct infusion of Ex-4 and regardless of hunger state (Dickson et al., 2012). Furthermore, HF-diet induced obesity appears to alter this system, as similar to the observations here, HF rats were less responsive to the anorexic effects of peripheral Ex-4 and lower fasting levels of GLP-1 (Williams et al., 2011). While the changes that occur to GLP-1 within the brain during obesity have yet to be explored, there is mounting evidence for GLP-1 as a modulator of hedonic eating and food reward via its actions in the mesolimbic dopamine pathway, with evidence for involvement of the VTA being particularly strong.

**IMPLICATION FOR GLP-1: CLINICAL RELEVANCE AND CONCLUSIONS**

As stated previously, the obesity epidemic demands greater understanding of what drives food choices and feeding behaviors, the changes that occur in the obese state, and how those changes influence behavior. Among these include critical changes to various hormones, such as GLP-1, and signaling with the brain in sites of reward such as the VTA. Collectively, this data suggests that GLP-1 is an important modulator of food reward, likely exerting its role through actions in the brain, particularly the mesolimbic dopamine system. Furthermore, we found that while obesity can influence the effectiveness of GLP-1 to reduce
hedonic eating, GLP-1R agonists similar to those already shown to be effective and safe for regulating blood glucose levels can also potently reduce sucrose intake. When testing how GLP-1R stimulation influenced motivation for rewarding food, the results found here, and from those in other laboratories have found that both central and peripheral treatments of GLP-1R agonists can significantly reduce the motivation to work for and consume a sweet reward.

As the GLP-1 system appears to be significantly altered following RYGB surgery concurrently with changes in taste and food choices, researchers have questioned if changes in GLP-1 and other hormones may a factor in the success of the surgery. Our data, along with that of others, suggests that GLP-1, alone or in combination with other hormones, could serve as a pharmacotherapeutic target and alternative to surgical procedures. However, further research into the clinical implications are needed, but the current literature strongly suggests a potential role for GLP-1 in controlling the complex set of behaviors regarding food choice and intake.


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