CENTRAL ACTIONS OF GLUCAGON-LIKE PEPTIDE-1 ON FOOD INTAKE AND REWARD: NOVEL NEUROLOGICAL TARGETS AND SEX DIVERGENT EFFECTS

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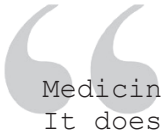

Medicine is not only a science; it is also an art. It does not consist of compounding pills and plasters; it deals with the very processes of life, which must be understood before they may be guided.

-Paracelsus

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ABSTRACT

Obesity is one of the biggest health risks of our society today; however, treatment options are sparse and most pharmaceutical manipulations result in suboptimal weight-loss outcomes. The development of more effective treatment options for this disease is therefore crucial. The glucagon-like peptide-1 (GLP-1) receptor (GLP-1R) agonist liraglutide was recently approved for the treatment of obesity in the US. GLP-1, and synthetic analogues of the peptide, reduce body weight by suppressing food intake and food reward through actions on GLP-1Rs in the central nervous system. The regulation of homeostatic feeding by GLP-1 was previously thought to be mediated through actions within the hypothalamus, while its effects on food reward were attributed to actions within the limbic system. Our studies challenge this view and demonstrate novel central areas which mediate the effects of GLP-1R stimulation on food intake and reward.

Using standard food intake and body weight measurements, in addition to tests of reward behavior, such as the operant conditioning and conditioned place preference tests, we demonstrate that GLP-1R stimulation, using the GLP-1R agonist exendin-4 (Ex4), reduces food intake and food reward behavior through actions in the nucleus of the solitary tract (NTS) and lateral hypothalamus (LH). Using a transgenic mouse line expressing fluorescent YFP-preproglucagon neurons, NTS GLP-1 neurons were found in close proximity to noradrenergic neurons, providing a potential connection to the mesolimbic system. Intra-NTS Ex4 injection also led to an increase in dopamine-related genes in the ventral tegmental area; further suggesting a link between the NTS and the reward system in which GLP-1 can alter reward-related behavior. In addition, the parabrachial nucleus (PBN) was identified as a novel area mediating the anorexic effects of GLP-1R stimulation.

Sex differences have been implicated in the regulation of reward, and the sensitivity of several ingestive hormones has been shown to differ between

males and females. However, potential differences in the actions of GLP-1 on food reward were previously unknown. Central GLP-1R stimulation led to increased suppression in food-motivated behavior in females compared to males. In addition, central estrogen blockade, and blockade of estrogen receptor-α (ERα) specifically, attenuated the effects of Ex4 on food reward, but not food intake. Therefore, these data suggest that central ERα signaling is necessary for the actions of GLP-1 on food-reward behavior in both sexes, while females display a much higher sensitivity to the food reward impact of central GLP-1R activation. Moreover, we also show that the actions of intra-LH GLP-1R stimulation on food-reward behavior are regulated in a sex divergent manner, where GLP-1R stimulation is sufficient to reduce food-motivated behavior in both sexes, but only necessary in males. In addition to food reward, Ex4 treatment in the LH also induced a robust reduction in food intake and body weight in a sex-dependent manner; chronic knockdown of LH GLP-1Rs, using an adeno-associated virus (AAV)-short hairpin RNA targeting GLP-1R transcripts, increased ingestive behavior and body weight in both sexes, but only increased food-motivated behavior in males.

In conclusion, the effects of GLP-1, and its synthetic agonists, on food intake and food reward are not bound to actions on GLP-1R exclusively within homeostatic or hedonic feeding centers, respectively. In contrast, GLP-1 can also exert its actions on food reward by acting in classic homeostatic centers, such as the NTS and the LH. In addition, a novel site of action was identified for GLP-1's actions on food intake: the PBN. Furthermore, GLP-1-mediated food reward, but not food intake, suppression is dependent on estrogen signaling, with a higher sensitivity to its actions in females. However, GLP-1 may also act differently within specific brain nuclei to regulate food-motivated behavior, as LH GLP-1R stimulation is sufficient to reduce food-reward in both sexes, while it only seems to be necessary for its actions in males.

Keywords: Glucagon-like peptide-1, Food reward, Food intake, Sex differences.

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POPULÄRVETENSKAPLIG SAMMANFATTNING

Övervikt och fetma är växande folkhälsoproblem, både i Sverige och i resten utav världen. Tillståndet är även ofta associerad med andra åkommor, såsom en ökad risk för hjärt- och kärlsjukdomar, typ 2 diabetes, och särskilda cancerformer. Trots att andelen överviktiga nästan fördubblats under de senaste 20 åren finns ännu ingen effektiv behandling mot denna sjukdom.

År 2014 godkändes läkemedlet liraglutide för behandling av övervikt i USA på grund av dess aptitdämpande och viktminskande effekter. I Sverige, och övriga delar av världen, används det främst för behandling av typ II diabetes på grund av dess blodsockerreglerande egenskaper. Liraglutide är en syntetisk variant av det kroppsegna hormonet glucagon-like peptide-1 (GLP-1), som frisätts från tarmen vid födointag och bidrar till en ökad mättnadskänsla genom att verka på det centrala nervsystemet. GLP-1 kan även produceras lokalt i hjärnan, främst i nucleus of the solitary tract (NTS).

Utöver dess aptitdämpande effekt, har GLP-1 även visat sig kunna inverka på hjärnans belöningssystem för att minska den belönande upplevelsen av föda, särskilt vid intag av kaloririka livsmedel med hög andel fett och socker. Trots att läkemedel som innehåller syntetiska varianter av detta hormon används flitigt runtom i världen, är mekanismerna bakom dess aptit- och belöningsdämpande effekter ännu inte fullt utredda. Dessutom har dess effekter och specifika mekanismer ej utretts i honor/kvinnor, trots indikationer att kvinnor reglerar både födointag och belöning på ett annorlunda sätt än män. Vår forskning ämnade därför utreda de specifika hjärnområdena och mekanismer som ligger bakom de aptit- och belöningsdämpande effekterna av GLP-1 och GLP-1-baserad behandling, samt undersöka potentiella könskillnader i dessa effekter. Dessa punkter studerades med hjälp av djurexperimentella modeller.

Med hjälp av det GLP-1-baserade läkemedlet exendin-4 (Ex4) identifierade vi två nya hjärnområden som förmedlar hormonets/läkemedlets effekter på födo-associerad belöning: NTS och lateral hypothalamus (LH), samt ett nytt område som reglerar dess matintagsdämpande effekter: parabrachial nucleus (PBN). NTS och LH är klassiska födointagsrelaterande center, men GLP-1s belöningsreducerande effekt i dessa områden var tidigare okända. För att undersöka hormonets påverkan på dessa effekter använde vi oss utav två klassiska belöningstest: operant betingning (operant conditioning) och konditionerad plats preferens (conditioned place preference; CPP). Testen mäter motivation för att erhålla en belöning (i detta fallet belönande föda), samt utvärderar drogens förmåga att påverka matens belönande egenskaper. Dessutom mättes djurens kroppsvikt och födointag av vanlig och belönande föda i respons till Ex4 behandling. Genom att använda oss av transgena möss kunde vi även påvisa förekomsten av GLP-1 fibrer i dessa områden, vilket tyder på att förutom läkemedel, kan kroppseget GLP-1 även verka i områdena. Man

fann även att GLP-1 nervceller i NTS med stor sannolikhet är kopplat till, och påverkar, belöningssystemet.

GLP-1s effekter på födo-associerad belöning visade sig även vara beroende av könshormonet östrogen. Östrogen bildas i könskörtlarna hon kvinnor (äggstockarna) och män (testiklarna), samt i hjärnan. GLP-1 behandling verkade belöningsdämpande i både honor och hanar, dock i högre grad i honor; denna effekt visade sig även förmedlas via östrogen receptor alpha (Erα). Man fann inga skillnader i Ex4s effekter på födointag. Vidare fann man även att GLP-1s belöningshämmande effekter på mat regleras annorlunda i honor och hanar specifikt i hjärnområdet LH. Stimulering av GLP-1 receptorer i LH reducerade födoämnesbelöning i båda könen; dock är hormonets effekter på det här beteendet nödvändig i hanar och blockering av de här receptorerna påverkar därför inte födoämnesbelöning i honor. Man fann inga könsskillnader på matintag efter Ex4 behandling i LH.

Sammanfattningsvis identifierar forskningen i denna avhandling två nya områden som medverkar i de reducerande effekterna av GLP-1 på födointag och födoassocierad belöning: NTS och LH, samt ett område som förmedlar hormonets effekter på matintag: PBN. Forskningen ökar även kunskapen om ett flertal mekanismer genom vilka GLP-1 kan förmedla dess kroppsviktsreglerande effekter. Dessutom fann vi även könskillnader i hormonets effekt på födoämnesbelöning, medan regleringen av matintag inte påverkades av kön, eller könshormonet östrogen. Våra fynd bidrar därmed till utvecklingen av mer effektiva behandlingsmetoder mot övervikt, samt bättre insyn i de mekanismer som existerande GLP-1-baserade behandlingar verkar igenom. Fynden tyder även på att läkemedel av denna sort kan behöva anpassas beroende på kön för att säkerställa effektiv behandling samt undvika onödiga biverkningar.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. ACTIVATION OF THE GLP-1 RECEPTORS IN THE NUCLEUS OF THE SOLITARY TRACT REDUCES FOOD REWARD BEHAVIOR AND TARGETS THE MESOLIMBIC SYSTEM.
Richard JE, Anderberg RH, Göteson A, Gribble FM, Reimann F, Skibicka KP.
PloS One. 2015 Mar 20;10(3):e0119034.

II. GLP-1 RECEPTOR STIMULATION OF THE LATERAL PARABRACHIAL NUCLEUS REDUCES FOOD INTAKE: NEUROANATOMICAL, ELECTROPHYSIOLOGICAL, AND BEHAVIORAL EVIDENCE.
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III. SEX AND ESTROGENS ALTER THE ACTION OF GLUCAGON-LIKE PEPTIDE-1 ON REWARD.
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INTERLEUKIN-6 (IL-6) IN THE CENTRAL AMYGDALA IS BIOACTIVE AND CO-LOCALIZED WITH GLUCAGON-LIKE PEPTIDE-1 (GLP-1) RECEPTOR.
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CONTENT

ABBREVIATIONS

5TG	5-thio-D-glucose
AAV	Adeno-associated virus
aCSF	Artificial cerebral spinal fluid
AgRP	Agouti-related peptide
ANOVA	Analysis of variance
AP	Anterior/posterior
ARC	Arcuate nucleus
BMI	Body Mass Index
Cal	Calorie(s)
CCK	Cholecystokinin
cDNA	Complementary DNA
CNS	Central nervous system
CPP	Conditioned place preference
CSF	Cerebral spinal fluid
CTB	Cholera Toxin Subunit B
DMH	Dorsomedial hypothalamus
DMSO	Dimethyl sulfoxide
DPP-IV	Dipeptidyl-peptidase IV
DV	Dorsal/ventral
ERα	Estrogen receptor-α
ERβ	Estrogen receptor-β
Ex9	Exendin-3(9-39)
FISH	Fluorescent in situ hybridization
FR	Fixed ratio
FSH	Follicle-stimulating hormone
GABA	γ-Aminobutyric acid
GHS-R	Growth hormone secretagogue receptor
GLP-1	Glucagon-like peptide-1
GLP-1R	GLP-1 receptor
ICI	ICI 182, 780
Ig	Immunoglobulin
J	Joule
k	Kilo
kcal	kilocalorie(s)
kJ	Kilojoule
L-DOPA	L-dihydroxyphenylalanine

LH	Lateral hypothalamus
lPBN	Lateral parabrachial nucleus
LuH	Luteinizing hormone
MCH	Melanin-concentrating hormone
ML	Medial/lateral
MPPd	MPP dihydrochloride
mRNA	Messenger RNA
NAc	Nucleus accumbens
NPY	Neuropeptide Y
NTS	Nucleus of the solitary tract
OB-R	Leptin receptor
PBN	Parabrachial nucleus
PFC	Prefrontal cortex
POMC	Proopiomelanocortin
PPG	Preproglucagon
PPIA	Peptidylprolyl isomerase A
PVN	Paraventricular nucleus
PYY_{3-36}	Peptide YY_{3-36}
RT-qPCR	Quantitative reverse transcription PCR
scp	Superior cerebellar peduncles
shRNA	Short hairpin RNA
TH	Tyrosine hydroxylase
VMH	Ventromedial hypothalamus
VTA	Ventral tegmental area
WHO	World Health Organization
YFP	Yellow fluorescent protein

INTRODUCTION

OVERWEIGHT AND OBESITY

Obesity is a chronic medical condition that currently kills more people worldwide than underweight and undernutrition. The condition also increases the risk for other serious diseases such as type II diabetes, cardiovascular diseases and cancer. According to the World Health Organization (WHO) more than 1.9 billion adults were overweight worldwide in 2016, and over 650 million were obese.

Obesity is primarily attributed to excessive energy intake, often through the intake of high-fat/high-sugar foods, which exceeds energy expenditure. Excess energy is stored as lipids in the adipose tissue in various fat depots around the body, and its regulation and organization can differ based on sex.

Energy intake refers to the amount of energy that we ingest through food (in the form of proteins, carbohydrates and fat), and is commonly measured in calories (cal) or joule (J). The standard daily recommended energy intake for women is 2000 kcal (8400 kJ), and 2500 kcal (10500 kJ) for men. Energy expenditure is determined mainly by the energy required to uphold our resting metabolic rate, and the energy that we expend through physical activity.

An individual's body mass state can roughly be determined using the body mass index (BMI), which is calculated by dividing an individual's body weight in kilograms (kg) by the square of their height in meters (m; BMI (kg/m^2) = mass/ $height^2$). Commonly, an individual with a BMI lower than 18.5 kg/m^2 is considered underweight, between 18.5 and 25 kg/m^2 normal weight, 25-30 kg/m^2 overweight and >30 kg/m^2 obese.

Energy intake is driven by our feeding behavior, which is controlled by the homeostatic and hedonic food intake regulating systems. These systems are coordinated through an intricate interplay of anorexic and orexigenic peptides which act within the gut, as well as in the central nervous system (CNS).

FOOD INTAKE AND BODY WEIGHT REGULATION

THE HOMEOSTATIC SYSTEM

Appetite and body weight are regulated by two intertwined neural pathways: homeostatic and hedonic (Berthoud, 2011; Saper et al., 2002). The homeostatic system ensures that energy balance is maintained; i.e. eating when energy stores are depleted, and refraining from eating when adequate energy is present. This process is regulated by an intricate array of anorexigenic (e.g. leptin, insulin, glucagon-like peptide-1 (GLP-1), estradiol) and orexigenic (e.g. ghrelin, neuropeptide-Y (NPY), agouti-related peptide (AgRP)) molecules produced in the periphery and within the central nervous system (CNS) (*Figure 1*). These peptides will be discussed briefly below.

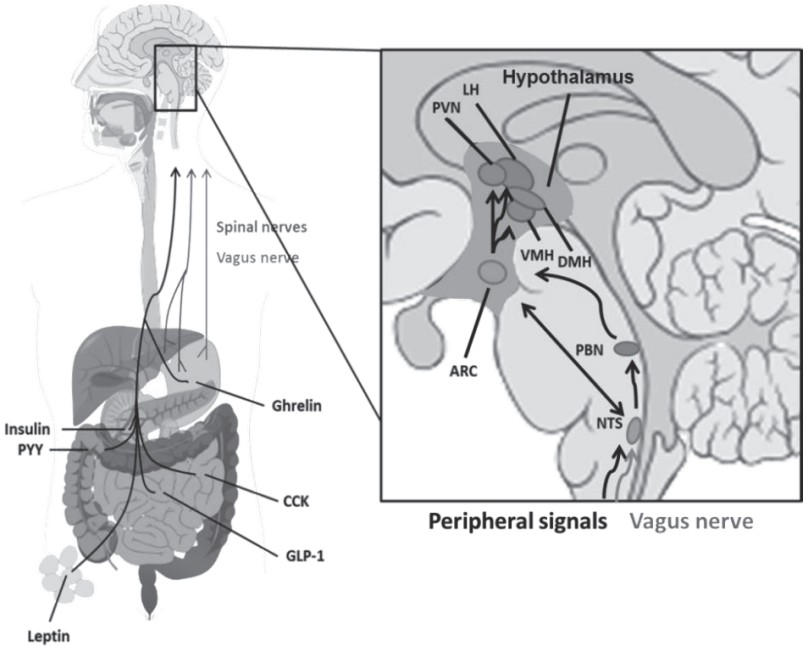

Figure 1. Representative image of peripheral signals and brain areas involved in the homeostatic regulation of food intake. PYY = peptide YY, CCK = cholecystokinin, GLP-1 = glucagon-like peptide-1, PVN = paraventricular nucleus, LH = lateral hypothalamus, VMH = ventromedial hypothalamus, DMH = dorsomedial hypothalamus, PBN = parabrachial nucleus, NTS = nucleus of the solitary tract. Image modified after composing illustrations from Wikimedia Commons and Public Domain Files.

MOLECULES INVOLVED IN FOOD INTAKE REGULATION

GHRELIN

Ghrelin, "the hunger hormone", was discovered in 1999 by Kojima *et al.* (Kojima et al., 1999). Circulating ghrelin levels are high during fasting, and levels rise in response to weight loss (Ariyasu et al., 2001; Cummings et al., 2004; Yoshimoto et al., 2002).

Ghrelin is produced in the stomach; it is released in the hunger state and stimulates feeding by acting on its receptor, the growth hormone secretagogue receptor (GHS-R) located within the arcuate nucleus (ARC), on NPY/AgRP neurons. These neurons are inhibitory and synapse on POMC neurons which inhibit food intake through the synthesis of melanocortin peptides; ghrelin therefore acts to promote feeding by removing these inhibitory signals (Nakazato et al., 2001) (*Figure 2*). In addition to stimulating food intake in response to hunger, ghrelin has also been suggested to play a role in stress-induced feeding. Rodents with increased caloric intake, due to chronic social defeat, display increased plasma ghrelin concentrations, and ghrelin secretion in humans is also increased in individuals prone to stress-induced feeding (Patterson et al., 2013; Raspopow et al., 2010, 2014). The increased caloric intake in response to stress is driven selectively by an increase in the intake of high carbohydrate-containing foods (Patterson et al., 2013; Schele et al., 2016). In addition to appetite, ghrelin also reduces fat utilization and increases adiposity (Tschop et al., 2000).

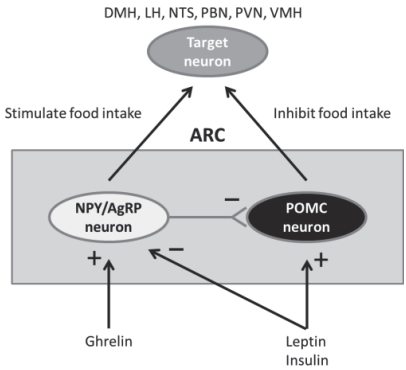

Figure 2. Schematic scheme of the regulation of food intake through the actions of hormones ghrelin, leptin and insulin on NPY/AgRP and POMC neurons. NPY = neuropeptide Y, AGRP = agouti-related peptide, POMC = proopiomelanocortin, DMH = dorsomedial hypothalamus LH = lateral hypothalamus, NTS = nucleus of the solitary tract, PBN = parabrachial nucleus, PVN = paraventricular nucleus, VMH = ventromedial hypothalamus.

LEPTIN

Leptin is a hormone secreted by the adipose tissue; it plays a key role in energy balance regulation by informing the brain of the body's energy storage level, as leptin is proportionately secreted in regard to body fat mass. It acts in a regulatory manner on body weight by limiting energy intake and promoting energy expenditure when adiposity is high (high circulating leptin), and promoting food intake, reducing energy expenditure and increasing fat accumulation when leptin levels are low (Cohen et al., 2001; Morton et al., 2006; Zhang et al., 1994).

Leptin regulates energy balance by acting on its receptor, OB-R, which is located within the hypothalamus, in areas such as the lateral hypothalamus (LH), paraventricular nucleus (PVN), ventromedial hypothalamus (VMH), and ARC (Morton et al., 2006). As for ghrelin, many of leptin's effects are mediated by actions on its receptors on NPY/AgRP and POMC neurons. However, contrary to ghrelin, leptin suppresses the activity of NPY/AgRP neurons and increases the activity of POMC neurons; increased synthesis of melanocortin peptides by POMC neurons therefore results in a reduction in food intake (Morton et al., 2006).

Besides the hypothalamus, leptin also acts within the hindbrain (in the nucleus of the solitary tract; NTS), and within several structures of the limbic system, such as the hippocampus, amygdala, ventral tegmental area (VTA) and LH to reduce food intake (Figlewicz et al., 2003; Kanoski et al., 2011b; Leinninger and Myers, 2008; Leshan et al., 2006; Suarez et al., 2019).

INSULIN

Insulin is secreted from pancreatic β-cells and is crucial in the regulation of energy and glucose homeostasis (Prentki et al., 2013). In addition to its peripheral effects on hepatic glucose production and secretion, insulin also acts within the brain to regulate glucose and energy homeostasis (Belgardt and Bruning, 2010). Like leptin, insulin is an anorexigenic hormone that acts within the brain to convey the body's adiposity level (Kennedy, 1953).

The effects of insulin on food intake and body weight are mainly attributed to its actions on insulin receptors located within the hypothalamus (Bruning et al., 2000; McGowan et al., 1992; Obici et al., 2002; Strubbe and Mein, 1977). Insulin receptor expression is high in the ARC, and its receptors can be found on both NPY/AgRP and POMC neurons (Benoit et al., 2002; Carvalheira et al., 2005). Leptin and insulin have been shown to act in concert to inhibit NPY/AgRP neurons and therefore reduce food intake.

CCK

CCK was the first anorexigenic gut hormone discovered (Gibbs et al., 1973). The hormone is secreted from cells within the duodenum and small intestine; it binds to CCK receptors on the vagus nerve terminal, which relays the information to the hypothalamus via the NTS and parabrachial nucleus (PBN) (Liddle et al., 1985). There are two different subtypes of CCK with distinct locations of expression; CCK-A is primarily expressed in the gastrointestinal tract, while CCK-B is primarily expressed in the CNS (Wank, 1995). Central CCK receptors can be found within the hippocampus, cerebral cortex, and striatum, in addition to the NTS (Beinfeld, 2001). The central actions of CCK on food intake are mainly attributed to its effects on receptors within the brainstem (Aja et al., 2001).

PEPTIDE YY_{3-36} (PYY_{3-36})

PYY is co-secreted with GLP-1 in the intestinal L-cells in response to food intake; it is rapidly metabolized in the circulation to PYY_{3-36} by dipeptidyl peptidase IV (DPP-IV), and acts to reduce food intake and body weight (Batterham et al., 2002). The effects of PYY_{3-36} on food intake are mainly attributed to the actions of the hormone within the hypothalamus; peripheral injection of PYY_{3-36} induces neuronal activation in the ARC, and decreases the expression of hypothalamic NPY mRNA. Furthermore, intra-ARC injection of PYY_{3-36} directly inhibits food intake by inhibiting NPY/AgRP neurons (Michel et al., 1998). Besides ARC, the PYY_{3-36} receptor, Y_2, is also expressed in the preoptic nucleus, dorsomedial hypothalamus (DMH), amygdala, substantia nigra, PBN and NTS (Dumont et al., 1998; Gustafson et al., 1997).

In addition to the food intake regulating hormones and molecules above, the anorexigenic peptide GLP-1 is also an important regulator of food intake and body weight. The role of GLP-1 in energy homeostasis will be discussed in further detail below.

BRAIN AREAS INVOLVED IN FOOD INTAKE REGULATION

Two of the classic brain areas involved in homeostatic food intake regulation, the hypothalamus and the brainstem, are located in close proximity to the brain's ventricles, which contain cerebrospinal fluid (CSF), and receive peripherally transferred food intake-regulating signals via areas characterized by more permeable blood-brain barriers, called circumventricular organs.

HYPOTHAMALUS

The hypothalamus has long been depicted as "the feeding center". Early studies by Anand and Brobreck demonstrated that lesioning the VMH led to a significant increase in food intake, while lesioning the ventral LH led to starvation and malnutrition (Anand and Brobeck, 1951a, b).

The LH is one of the most interconnected areas of the hypothalamus, receiving and sending projections to and from many important food intake regulating areas, such as the NTS, amygdala and nucleus accumbens (NAc), in addition to other hypothalamic nuclei (Berk and Finkelstein, 1982; Elias et al., 1999; Elias et al., 1998; Ricardo and Koh, 1978; Simerly, 1995; Ter Horst et al., 1989; Ter Horst and Luiten, 1987).

The LH contains three distinct neuronal cell types known to regulate food intake behavior: orexin/hypocretin, melanin-concentrating hormone (MCH) and neurotensin neurons. The LH is the sole area in the CNS that synthesizes and releases the orexigenic neuropeptide orexin; orexin increases food intake through its actions within the brain (Harrison et al., 1999; Sakurai, 1999). The effects of orexin are thought to be mediated partly through actions on its receptors in hypothalamic subregions, such as the DMH, and within the LH itself (Dube et al., 1999; Sweet et al., 1999). Orexin neurons also project to several other food intake-regulating areas, which also contain orexin receptors, such as the NTS (Hervieu et al., 2001; Marcus et al., 2001; Peyron et al., 1998; Zheng et al., 2005). Injection of orexin into the hindbrain has been shown to increase meal size, and intra-NTS injection selectively increases the intake of high-fat foods (Baird et al., 2009; Kay et al., 2014; Parise et al., 2011). The LH also contains MCH producing neurons, which project to a wide array of central areas, such as the striatum, thalamus, cerebral cortex, midbrain and brainstem (Bittencourt et al., 1992; Broberger et al., 1998). MCH is an orexigenic hormone; central injection of the peptide increases food intake and body weight (Qu et al., 1996). Furthermore, overexpression of MCH leads to hyperphagia and subsequently obesity, while knockout of the peptide leads to reduced food intake (Alon and Friedman, 2006; Shimada et al., 1998). Neurotensin neurons

also reside within the LH, and have been hypothesized to play role in energy balance regulation. Both peripheral and central administration of neurotensin reduce food intake; in addition, ablation of these neurons, or knockout of its receptor, leads to hyperphagia and obesity (Cooke et al., 2009; Kim et al., 2008; Leinninger et al., 2011).

The LH integrates a wide array of molecular signals that regulate food intake, such as glucose, insulin, leptin, ghrelin, GLP-1 and PYY_{3-36} (Berthoud and Munzberg, 2011).

Subsequent research has identified several other important food intake regulating hypothalamic nuclei, such as the ARC, PVN and DMH. As mentioned above ARC contains NPY/AgRP and POMC neurons, which act to stimulate, or inhibit, food intake through the actions of various hormones. The PVN also contains POMC neurons, and destruction of this area leads to overeating (Leibowitz et al., 1981). Furthermore, deletion of the hypothalamic nucleus DMH reduces food intake (Bellinger and Bernardis, 2002).

NTS

The NTS is located in the caudal brainstem, ideally positioned to mediate food intake regulating signals between the periphery and the CNS. The rostral region of the NTS sends gustatory signals to the forebrain, facilitating taste recognition, while the caudal NTS integrates viscerosensory information (Travagli et al., 2006). It receives afferent connections from the vagal nerve which innervates most of the gastrointestinal system, making it possible for the NTS to sense gastric distension, and rapidly release anorexic signals (such as leptin, CCK and GLP-1) in response to food intake (Andresen and Kunze, 1994; Cassidy and Tong, 2017).

The NTS contains several different neuronal cell types involved in food intake regulation, for instance catecholamine, POMC and GLP-1 neurons (Rui, 2013). NTS catecholamine neurons respond to anorexigenic and orexigenic hormones from the periphery, such as CCK, which activates these neurons to reduce food intake, and ghrelin, which inhibits catecholamine neurons to stimulate food intake (Appleyard et al., 2007; Cui et al., 2011). In addition to catecholaminergic neurons, CCK can also act on POMC neurons within the NTS to reduce food intake (Fan et al., 2004). The NTS is also the major CNS producer of GLP-1; which, apart from the NTS, is only produced in a small population of interneurons in the olfactory bulb and in the intermediate reticular nucleus (Merchenthaler et al., 1999; Thiebaud et al., 2016; Vrang and Larsen, 2010). The actions of GLP-1 on food intake will be discussed in further detail below.

As an important integrator of peripheral and central signals, the NTS mediates the energy balance effects of a wide array of vagal and endocrine signals and, in addition to integrating them, relays them to other important central food intake regulating areas, such the hypothalamus and PBN. In addition it also projects to reward-related areas such as the NAc and the VTA, both through direct projections or via the hypothalamus (Alhadeff et al., 2012; Travagli et al., 2006), which creates a neuroanatomical pathway for direct brainstem influence on reward behaviors.

PBN

Of the many brain areas which receive connections from the NTS, the PBN is one of its major targets, relaying information to other food intake regulating brain areas, such as the hypothalamus and amygdala (de Araujo, 2009; Herbert and Saper, 1990; Jhamandas and Harris, 1992; Palmiter, 2018; Wu et al., 2012).

The PBN, located within the dorsolateral pons, integrates viscerosensory information, such as satiety, malaise and taste (Berridge and Pecina, 1995; Palmiter, 2018; Swank and Bernstein, 1994; Yamamoto, 2006).

Several neuropeptides act in this area to regulate feeding; for example, injection of melanocortin or prostaglandin agonists in the PBN leads to a reduction in food intake behavior (Skibicka et al., 2011a; Skibicka and Grill, 2009), while injection of cannabinoid or μ-opioid agonists increases feeding (DiPatrizio and Simansky, 2008; Wilson et al., 2003). In addition, disturbed balance of PBN input signals of γ-aminobutyric acid (GABA) and glutamate, the major excitatory and inhibitory neurotransmitters in the brain leads to starvation in mice (Carter et al., 2013; Wu et al., 2009; Wu and Palmiter, 2011; Wu et al., 2013).

SEX DIFFERENCES IN FOOD INTAKE REGULATION

More women than men are overweight and obese worldwide (Chooi et al., 2019). In addition, men and women have differential patterns of body fat distribution. While men primarily tend to accumulate fat viscerally, within the abdominal cavity, women commonly accumulate fat subcutaneously, around the buttocks, thighs and hips (Demerath et al., 2007; Kotani et al., 1994). This difference is abolished through ovariectomy, the removal of the ovaries; the main source of steroidal sex hormones in females (Simpson, 2003). In addition to a shift in the location of fat storage, removal of the ovaries also leads to a marked increase in adipose tissue, an effect mainly attributed to the reduction of the hormone estrogen (Stotsenburg, 1913).

Steroid hormones, such as estrogen, progesterone and testosterone, are all produced from cholesterol. Both men and women produce steroid hormones, albeit at different levels, and these hormones mediate various physiological functions in both sexes, such as reproduction, inflammation and metabolism. The gonads are the major source of these hormones; where the ovaries are the primary production site in females, and the testes the primary source in men (Baggett et al., 1959; Brook, 1999).

In women, the levels of specific gonadal steroid hormones vary over the course of approximately 28 days. This cycle, the menstrual cycle, is divided into 3 phases: follicular, periovulatory and luteal (*Figure 3*). The follicular phase begins with menstruation, where the levels of all four of the main female gonadal hormones, luteinizing hormone (LuH), follicle-stimulating hormone (FSH), estradiol and progesterone, are low. However the level of estradiol, the main estrogen, begins to rise during this cycle phase, reaching its peak in the periovulatory phase, where it dramatically drops, almost to baseline. The periovulatory phase is characterized by a surge in FSH and LuH, where the sudden surge in LuH is necessary for ovulation (the release of the egg or ovum) to occur. Following the LuH and FSH surges, estradiol and progesterone levels begin to rise, but decline again if fertilization hasn't occurred (the fusion of the egg and sperm) (Hawkins and Matzuk, 2008).

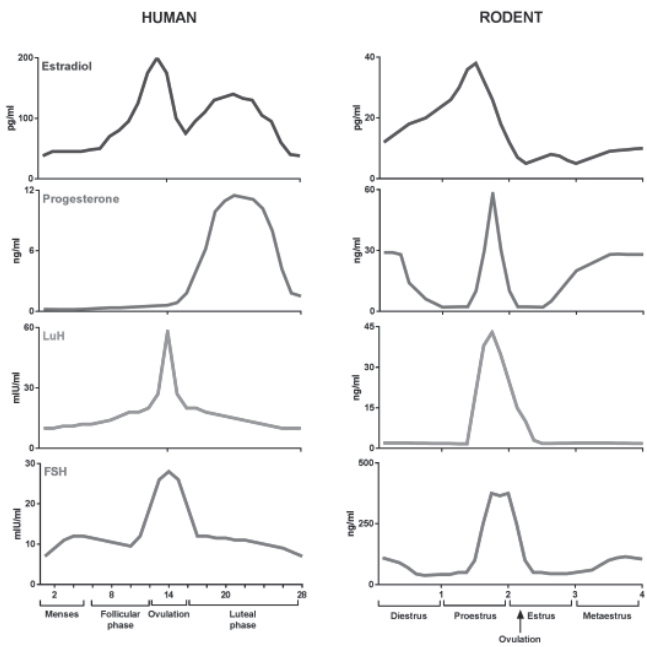

Figure 3. Representative plots of the human menstrual cycle and rat estrous cycle phases. Each phase is characterized by different levels of the gonadal hormones estradiol, progesterone, luteinizing hormone (LuH) and follicle-stimulating hormone (FSH). Figure derived from (Donner and Lowry, 2013).

In rodents, the estrous cycle is comprised of 4 cycle phases: diestrus (or diestrus II), proestrus, estrus and metaestrus (or diestrus I), which take place during a 4-5 day time period (*Figure 3*). As in the human follicular phase, the levels of estradiol, LuH and FSH are low in diestrus; however, in rodents, progesterone levels are high at the beginning of diestrus, and lfall prior to proestrus. Estradiol levels are also on the rise during this phase. All four hormones reach their peak in proestrus, and subsequently fall to baseline in the estrus phase, during which ovulation occurs. The levels of estradiol, progesterone and FSH slowly begin to rise again during metaestrus (Asarian and Geary, 1999).

While the steroidal hormones involved in reproduction are mainly produced in the gonads, these hormones can also be produced in several other areas, such as the adipose tissue and within the brain (Mellon et al., 2001). Brain derived hormones are commonly referred to as neurosteroids and are involved in several biological functions, such as neural plasticity, learning, memory, and psychological disorders e.g. anxiety and depression (Engel and Grant, 2001).

Neurosteroids have been shown to alter feeding behavior, and estrogens play a key role in the regulation of food intake and body weight. As mentioned above, removal of the ovaries leads to a marked increase in body weight, an effect which can be counteracted by the injection of the estrogen β-estradiol (Drewett, 1973; Simpson, 2003; Wade, 1975). In addition to body weight, estrogens have also been shown to directly regulate food intake. Food intake varies due the fluctuating levels of estradiol during the ovarian cycle, with reduced food intake in cycle phases where estrogen signaling is high (Czaja and Goy, 1975; Eckel, 2004; Gong et al., 1989; Houpt et al., 1979). Estrogens' effects on food intake and body weight are mediated by actions on the estrogen receptor (ER). There are two main types of nuclear estrogen receptors: estrogen receptor-α (ERα) and estrogen receptor-β (ERβ) (Deroo and Korach, 2006; Nilsson and Gustafsson, 2011). Activation of nuclear ER leads to binding of estrogen-response elements, which further bind to DNA to affect gene expression; a process which can take hours to days (Cheskis et al., 2007; Heldring et al., 2007). In addition, ERs can also be expressed outside the nucleus, on the cell membrane (Mendelsohn and Karas, 2010; Vasudevan and Pfaff, 2007). ER signaling kinetics can react to incoming stimuli from paracrine, autocrine and endocrine signals.

ERs play an important role in the homeostatic regulation of body weight. Whole body knock-out of ERα leads to increased body weight in both male and female mice (Heine et al., 2000). Moreover, specific knock-down of ERα in the VMH leads to increased body weight, hyperphagia, glucose intolerance and reduced energy expenditure (Musatov et al., 2007).

Estrogens can also affect food intake and body weight by interacting with other neuropeptides. For instance, females display increased sensitivity to the anorexic actions of leptin, an effect mainly attributed to the actions of estrogens (Clegg et al., 2006; Clegg et al., 2003). In addition, ghrelin increases food intake significantly more in males and ovariectomized females, than in intact females or females receiving estrogen replacement therapy, suggesting an inhibitory effect of estrogen on the actions of ghrelin (Clegg et al., 2007; Lopez-Ferreras et al., 2017). However, site specific injection of ghrelin in the LH increases body weight only in females, though food intake is increased in both sexes, indicating site-specific sex differences in the regulation of ghrelin's effects on food intake and body weight regulation (Lopez-Ferreras et al., 2017). Estrogen has also been shown to modulate the effects of CCK by increasing CCK-mediated satiation in intact females in phases of the estrous cycle with high circulating estrogen levels (Asarian and Geary, 1999, 2002; Eckel and Geary, 1999; Wager-Srdar et al., 1987).

THE HEDONIC SYSTEM

In addition to the homeostatic system, the body maintains an additional system to ensure that an individual strives to consume an adequate amount of food and nutrients: the hedonic system. While reward-driven eating was initially crucial for our survival, it has in recent decades become a problem due to the increasing accessibility of palatable, highly-caloric foods. Disinhibited intake of these food types can lead to overconsumption, weight gain and obesity. Food reward behavior is typically divided into two components: "liking" and "wanting", originally described by Berridge *et al.* (Berridge, 1996; Berridge et al., 2009). Liking is associated with the palatability of the food, and the immediate response to their consumption, while wanting is associated with the motivation to obtain a certain type of food.

THE MESOLIMBIC SYSTEM

The reward system drives us to pursue behaviors that result in rewarding and pleasurable feelings; these behaviors provide positive reinforcement, increasing the likelihood that the behavior will be repeated. Initial experiments by Olds and Milner identified several brain reward areas. When electrical probes were placed in distinct brain nuclei, animals would continuously self-stimulate, suggesting a rewarding effect of the stimulation in these areas (Olds and Milner, 1954). The mesolimbic pathway is a fundamental part of the reward system; it originates in the VTA, an area within the midbrain which sends dopaminergic projections to several areas within the limbic forebrain, such as the NAc, amygdala and hippocampus, in addition to the prefrontal cortex (PFC) (Dahlstrom and Fuxe, 1964; Koob, 1992; Nestler, 2004; Swanson, 1982) (*Figure 4*). In turn, the PFC sends projections to the NAc and VTA, creating a possibility for a loop-like feedback system (Scofield and Kalivas, 2014).

The NAc is a heterogenous structure; its two major subregions include the shell and the core, which have been shown to play dissociable roles in food reward regulation (West and Carelli, 2016; Zahm and Brog, 1992). Both structures receive dopaminergic inervation from the VTA, but project to different central areas. While the core mainly projects to motor structures, such as the cingulate motor areas and the premotor cortex, the shell mainly projects to other reward-associated structures such as the amygdala and LH, in addition to the brainstem (Salgado and Kaplitt, 2015). Moreover, while the NAc core seems to play an important role in reward learning, the shell plays an important role in the control of food reward (Kelley, 2004).

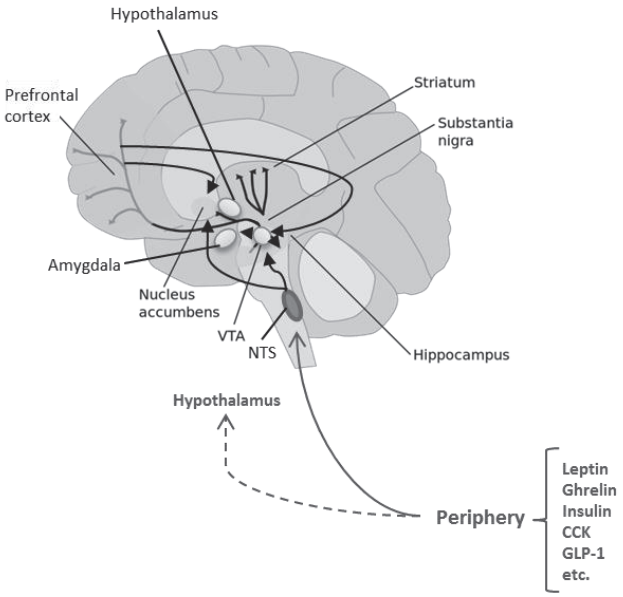

Figure 4. Representative image of the limbic system, and peripheral signals which alter food reward. Dopaminergic neurons project from the ventral tegmental area (VTA) to the nucleus accumbens (NAc), amygdala, hippocampus and prefrontal cortex. The VTA and nucleus accumbens receive returning projections from the prefrontal cortex. The nucleus of the solitary tract (NTS) projects to the two major reward areas, the VTA and the NAc. Image modified; original image acquired from Wikimedia Commons.

Initial studies on the reward system focused on its role in drug addiction (Berke and Hyman, 2000; Wise, 1996). However, later studies have shown that food and drugs affect many of the same reward areas. In fact, both drugs of abuse and palatable foods increase dopamine release in the brain reward system (Volkow et al., 2013). Dopamine is a catecholaminergic neurotransmitter involved in a variety of different physiological functions; in addition to reward it also plays a role in movement and emotional regulation. It is produced in the VTA, the midbrain and within the ARC, from the amino acid tyrosine, an enzyme derived from the liver. After synthesis, tyrosine is transported to dopaminergic neurons in the brain where it is converted to dopamine through several steps. The rate-limiting step is dependent on the actions of the enzyme tyrosine hydroxylase (TH) which converts tyrosine to L-dihydroxyphenylalanine (L-DOPA). L-DOPA is then converted to dopamine (Molinoff and Axelrod, 1971). Dopamine's role in food reward is based on original studies evaluating the effects of depletion of dopaminergic neurons using the neurotoxin 6-hydroxydopamine; depletion of these neurons led to a reduction in food intake and body weight in the animals (Ungerstedt, 1968, 1971; Zigmond and Stricker, 1972). In addition, dopamine receptor blockade using the antipsychotic drug

pimozide attenuates reward-driven food intake (Wise and Colle, 1984; Wise et al., 1978a; Wise et al., 1978b). Furthermore, genetic deletion of TH leads to hypophagia; an effect which can be counteracted by daily injections of L-DOPA (Zhou and Palmiter, 1995).

Apart from affecting the dopaminergic pathway, through taste or palatable food cues, food can also affect food reward through the actions of various hormones and signals secreted from the gastrointestinal tract in response to food intake (Alonso-Alonso et al., 2015). In fact, several of the hormones that participate in the homeostatic regulation of food intake also act on the reward system to alter food reward behavior.

MOLECULES INVOLVED IN FOOD REWARD REGULATION

LEPTIN AND GHRELIN

Leptin and ghrelin both act on the dopaminergic system to modulate food reward in an opposing manner. For instance, leptin administration in the VTA decreases firing of dopaminergic neurons in NAc, which leads to reduced food reward, and OB-R knock-down in the VTA increases dopamine release and sucrose preference (Hommel et al., 2006; Krugel et al., 2003). In contrast, ghrelin administration in the VTA or NAc increases food reward (Egecioglu et al., 2010; King et al., 2011; Perello et al., 2010; Skibicka et al., 2011b; Skibicka et al., 2012).

INSULIN

Insulin also alters dopamine release in the NAc; its effects are bidirectional, increasing dopamine release at low concentrations, while inhibiting release at higher concentrations (Potter et al., 1999). Furthermore, intraventricular insulin administration reduces the motivation to work for sucrose in rats (Figlewicz et al., 2006).

PYY_{3-36}

Though the effects of PYY_{3-36} on food intake are thought to be mediated by receptors within the ARC, receptors are also found throughout other areas of the brain, including reward related structures, such as the PFC, VTA and amygdala (Batterham et al., 2007). In addition, PYY_{3-36} has been shown to modulate resting activity in the brain reward system in humans, and co-administration of the peptide with GLP-1 additively reduces BOLD signal in response to food intake pictures in the amygdala and NAc in nonobese subjects (Batterham et al., 2007; De Silva et al., 2011). The effects of PYY_{3-36} on food

reward appear to be independent of actions of the peptide on Y_2 receptors in the ARC. Systemic PYY_{3-36} administration reduces high-fat food seeking; a behavior which is unchanged after intra-ARC PYY_{3-36} administration (Ghitza et al., 2007). In addition, PYY_{3-36} may play a role in the development of obesity, as postprandial levels of the peptide are lower in obese individuals, which may lead to reduced satiety and increased food intake (le Roux et al., 2006).

LH AND MOTIVATED BEHAVIOR

In addition to the NAc and VTA, the LH was identified long ago as an important regulator of motivated behavior. In fact, the LH is the most potent self-stimulation center in the brain. In regard to food reward, neuronal activation within the LH stimulates food intake and food seeking, even in satiated rats (Miller, 1960). Furthermore, hungry or food restricted rats self stimulate more than fed rats, suggesting a role for the LH in the interaction between metabolic status and reward (Blundell and Herberg, 1968; Margules and Olds, 1962). The LH also contains several receptor populations for important food intake regulating hormones and peptides, for example ghrelin, leptin and GLP-1; potential roles for these molecules on food reward in the LH are however largely unexplored (Berthoud, 2011; Berthoud and Munzberg, 2011).

NTS AND FOOD REWARD

Though not commonly considered a reward area, the NTS receives a number of peripheral and central signals affecting food reward. For example leptin, which has previously been shown to reduce food reward through its actions on the mesolimbic system, reduces the rewarding effects of food by acting on receptors in the medial NTS (Kanoski et al., 2014). In addition, orexin may be released in the NTS to increase food reward (Kay et al., 2014; Peyron et al., 1998; Zheng et al., 2005). The NTS is also the primary source of production of the hormone GLP-1, which has previously been implicated in food reward regulation. However, potential effects of GLP-1 on food reward in this area were previously unknown.

SEX DIFFERENCES IN FOOD REWARD

In addition to sex differences in food intake, differences have also been implicated in food reward. For instance, women have been shown to have more difficulty inhibiting their desire to eat when elicited by a food stimulus, and have an increased tendency to overeat when presented with palatable foods (Hays and Roberts, 2008; Wang et al., 2009).

Besides "homeostatic areas" regulating food intake, ERs can also be found in regions involved in reward regulation, such as the VTA and NAc, and estrogens have previously been shown to act within these areas to regulate reward (Shughrue et al., 1997). In the VTA, estrogens have been shown to cause functional changes in GABAergic neurons, and estrogen treatment leads to enhanced striatal dopamine release (Becker, 1990; Becker and Ramirez, 1981; Becker and Rudick, 1999; Dazzi et al., 2007; Febo and Segarra, 2004; McEwen and Alves, 1999; Zhang et al., 2008). Although little is known of the effects of estrogens on food reward behavior, sex and estrogens have previously been shown to play a role in the rewarding effects of drugs of abuse. For instance, estrogen administered in ovariectomized rats increases dopaminergic cocaine sensitivity, and estradiol administration during the follicular phase increases the subjective effects of amphetamine in women (Justice and De Wit, 2000; Zhang et al., 2008). Dopamine levels, levels of its metabolites and synthesizing enzymes, and amphetamine -induced dopamine release vary based on ovarian cycle phase (Becker, 1990; Becker et al., 1984). In addition, women progress faster to cocaine dependence, and display higher preference for cocaine rewards, effects potentially mediated by the actions of estrogens (Kerstetter et al., 2012; Kerstetter and Kippin, 2011).

Taken together, since estrogens modulate food intake, ERs are expressed in key reward-regulating areas, and there are sex differences in reward-regulated behaviors, we hypothesized that estrogen may also play a role in the regulation of the rewarding effects of food, specifically by modulating the rewarding effects of the peptide GLP-1.

CURRENT WEIGHT-LOSS TREATMENT OPTIONS

Obesity treatment primarily involves the application of lifestyle changes, including dietary changes, increased physically activity and behavioral therapy. These recommendations are often difficult to maintain and adherence to this treatment regimen is low, resulting in no more than 5-10% body-weight loss (Wadden et al., 2012).

Bariatric surgery, which is currently the most effective treatment for obesity, includes gastric bypass (Roux-en-Y), gastric banding or gastric sleeve, which involve reducing the size of the stomach mechanically or surgically. These procedures lead to a reduction in the capacity of the stomach, reducing the amount of food that can be consumed, in addition to decreasing energy and nutrient absorption. Gastric bypass results in an initial weight-loss of 30% within the first year, with 20% maintained weight loss up to 10 years after surgery (Maciejewski et al., 2016). However, bariatric surgery is invasive, and can result in acute or life-long side-effects, including bleeding, abdominal pain, chronic nausea and/or vomiting, excess loose skin, bowel destruction, ulcers and anastomotic stricture (Karmali et al., 2010). In addition, the treatment poses substantial economic costs on both the individual and society, making it a suboptimal treatment choice for obese individuals in general.

Apart from bariatric surgery, a number of pharmaceutical treatments can be prescribed for weight-loss. The following are the most commonly prescribed weight loss treatments available:

Orlistat (Xenical) acts locally within the gut to inhibit fat by binding to the enzyme lipase, thereby reducing lipid hydrolysis and absorption. Orlistat treatment results in an approximate 5% weight loss and potential side-effects include: increased flatulence, urgent bowel movements, inability to control bowel movements, stomach pain, rectal pain, nausea and vomiting (Jain et al., 2011).

Centrally acting pharmacological treatments include: Lorcaserin (Belvic), a serotonin 2C receptor agonist, phentermine-topiramate (Qsymia), the exact mechanism of which is unknown though it includes activation of hypothalamic noradrenergic neurons, and naltrexone-bupropion (Contrave), which reduces food intake by acting on POMC neurons in the hypothalamus. These drugs result in an approximate 5% reduction in body weight, and include a variety of side-effects ranging from gastrointestinal to psychological issues (Apovian et al.,

2013; Aronne et al., 2014; Greenway et al., 2009; Shin and Gadde, 2013; Thomsen et al., 2008). In addition, the synthetic GLP-1R agonist liraglutide (Saxenda) was also recently approved for weight-loss treatment. While the weight loss effects of this drug are slightly larger than orlistat or lorcaserin, liraglutide treatment only results in a 5-10% reduction in body weight (Mehta et al., 2017). Possible side effects of liraglutide treatment include nausea, diarrhea, constipation, abdominal pain, headaches and increased pulse. The effects of liraglutide on food intake and reward are mediated through actions on GLP-1Rs within the brain; however, the exact brain areas involved, and the mechanisms mediating these effects, are not fully known.

GLP-1 IN FOOD INTAKE AND REWARD

As mentioned above, GLP-1 has the ability to affect body weight regulation by acting on both the homeostatic and hedonic systems.

GLP-1 is a peptide hormone composed of 30 amino acids, produced and secreted in the intestinal L-cells. It is synthesized through enzymatic cleavage of preproglucagon (PPG), by prohormone convertase, in response to food intake (Donnelly, 2012; George et al., 1985; Mojsov et al., 1990; Novak et al., 1987). GLP-1 mediates its effects by binding to its receptor, the GLP-1R, a G-coupled receptor widely expressed throughout the periphery and the brain (Dunphy et al., 1998; Holst, 2007).

GLP-1 was first described as an incretin, stimulating insulin release and inhibiting glucagon secretion to regulate blood glucose (Kreymann et al., 1987; Orskov et al., 1988); it also acts in the gut to inhibit gastric emptying (Flint et al., 1998). Due to its glucoregulatory ability, synthetic GLP-1R agonists have been developed for the treatment of type II diabetes (Holst, 2004). Synthetic variants, such as exendin-4 (Ex4), liraglutide and dulaglutide, are resistant to degradation by GLP-1's primary metabolizer DPP-IV. By augmenting the site of cleavage, or using exendin-based treatments, the half-life of these GLP-1R acting compounds is significantly increased compared to endogenous GLP-1, which is degraded in a matter of minutes in the periphery (Vilsboll et al., 2003).

In addition to its role in blood glucose regulation, GLP-1 also has anorexigenic properties, as both central and peripheral administration of the peptide, or its synthetic variants, have been shown to reduce food intake and subsequently body weight (Abbott et al., 2005; Chelikani et al., 2005; Kanoski et al., 2011a; Tang-Christensen et al., 1996; Turton et al., 1996). Due to the weight-loss effects of GLP-1, GLP-1R agonist liraglutide was recently approved for weight management treatment for obese individuals (Mehta et al., 2017).

In addition to the gut, GLP-1 is also produced in the brain, primarily within the NTS as mentioned above (Merchenthaler et al., 1999). Centrally, GLP-1 acts to promote satiety by acting on GLP-1Rs in various brain regions. The effects of GLP-1 on satiety were initially attributed to the stimulation of GLP-1Rs in the hypothalamus, as site-specific injections of GLP-1 within this area decreases food intake, and peripheral injection of GLP-1R agonist has been shown to stimulate neuronal activity within the hypothalamus (Barrera et al., 2009; Goke et al., 1995; McMahon and Wellman, 1997, 1998; Pannacciulli et al., 2007; Turton et al., 1996). Hypothalamic nuclei that mediate GLP-1's effects on food

intake include the ARC, PVN, VMH, DMH and LH (McMahon and Wellman, 1998; Sandoval et al., 2008; Schick et al., 2003).

Besides the hypothalamic nuclei, GLP-1 has been shown to reduce food intake by acting on several other brain areas. For instance, GLP-1 is able to act locally within its central site of production: the NTS. GLP-1Rs are expressed throughout the NTS and site-specific injection of GLP-1 has been shown to reduce food intake (Hayes et al., 2009; Hayes et al., 2008). In addition, the PBN has been shown to contain GLP-1Rs and GLP-1 mRNA has been found in this area (Merchenthaler et al., 1999), however whether these receptors are important for food intake regulation was previously unknown.

Apart from homeostatic food intake regulation, GLP-1 has also been implicated in the regulation of food reward. Both systemic and central injection of GLP-1R agonist Ex4 reduces food reward behavior (Dickson et al., 2012). More specifically, GLP-1 has been shown to directly affect reward-related regions. GLP-1 neurons project from the NTS to the VTA and NAc, and GLP-1Rs are also expressed in these areas (Alhadeff et al., 2012; Goke et al., 1995; Merchenthaler et al., 1999; Rinaman, 2010). Moreover, GLP-1R agonist injection directly into the VTA and NAc inhibits food reward, and more specifically motivated behavior for palatable food (Dickson et al., 2012). In regard to the NAc, the effects of GLP-1 on food reward seem to be driven largely by its actions on GLP-1Rs in the NAc shell, while GLP-1Rs in the core only affect food intake regulation (Dickson et al., 2012; Dossat et al., 2011). GLP-1R stimulation in the NAc core does however influence palatability and the hedonic value of food, as blockade of GLP-1Rs in this area increases sucrose solution intake without affecting the intake of a non-nutritive saccharin solution (Dossat et al., 2013). Interestingly, GLP-1R stimulation in the VTA seems to specifically reduce the intake of rewarding foods, when both a palatable food choice and standard food choice (chow) are presented simultaneously (Alhadeff et al., 2012; Mietlicki-Baase et al., 2013). In addition to reward-regulating areas such as the NAc and the VTA, the LH is also innervated by GLP-1 neurons originating from the NTS, and contains GLP-1Rs (Merchenthaler et al., 1999). Interestingly, despite its strong connection with reward behavior, and its role in feeding behavior, whether GLP-1 acts within the LH to alter food reward was previously unknown.

Therefore, while initial studies of GLP-1's effects on food reward mainly focused on classic reward-regulating areas, the rewarding effects of the peptide on other central GLP-1R populations, and potential sex differences, were largely unexplored; these are two important topics which were investigated in this thesis.

AIMS

AIMS

The overall aim of this thesis was to investigate a potential role for GLP-1 on food reward in extra-mesocorticolimbic areas, and to investigate sex-dependent differences on the peptide's reward-regulating effects.

SPECIFIC AIMS

I. To investigate if GLP-1 can alter food reward by acting on GLP-1Rs directly within its primary CNS source of production: the NTS.

II. To explore if GLP-1 acts in the lateral PBN, a key nucleus in food intake control, to regulate the intake of standard and/or palatable foods, in addition to altering body weight.

III. To determine if there are sex-specific differences in the actions of GLP-1 on food reward behavior, and whether these differences are due to the actions of the steroid hormone estrogen.

IV. To investigate if GLP-1R stimulation within the LH, an important mediator of homeostatic and hedonic feeding behavior, is critical for body weight control, and if there are differences in the actions of LH-acting GLP-1 between males and females.

MATERIALS AND METHODS

ETHICS

All studies were carried out with ethical permissions from the Animal Welfare Committee of the University of Gothenburg (Decree 86/609/EEC). All efforts were made to minimize animal suffering.

ANIMALS

SPRAGUE DAWLEY RATS

Male (Paper I and II), or male and female (Paper III and IV), Sprague-Dawley rats were used in all behavioral experiments (purchased from Charles River, Germany). All animals were housed in a 12 hour light/dark cycle with *ad libitum* access to standard chow and water, unless otherwise specified.

YFP-PPG MICE

For GLP-1 detection studies (Paper I and II), adult male and female mGLU-124 Venus yellow fluorescent protein transgenic mice (YFP-PPG mice; University of Cambridge, United Kingdom) were used. Transgenic mice are mice in which their genome has been altered, often to better mimic a human state or disease. A new gene can be inserted or an existing gene knocked out, down, or up, to investigate its function. In this specific case a fluorescent gene was inserted to allow visualization of PPG-expressing cells. As mentioned in the introduction, PPG is the precursor peptide which is enzymatically cleaved to GLP-1 in response to food intake (Donnelly, 2012; George et al., 1985; Mojsov et al., 1990; Novak et al., 1987). PPG is also cleaved to other proteins, such as glucagon. However, glucagon expression in the brain is sparse compared to GLP-1, indicating that PPG cells most likely contain GLP-1 and not glucagon. In addition, cells were located in areas previously shown to contain GLP-1 neurons.

To generate the YFP-PPG mouse, a construct was first made in which the PPG promotor gene was coupled to a fluorescent gene (YFP). The genetic construct was then injected into a mouse embryo and integrated into the genome; YFP is then co-expressed in PPG-containing cells, making it possible to visualize the cell bodies and projecting fibers.

DRUGS

GLP-1 AND EX4

Endogenous GLP-1 has a very short half-life, lasting only a few minutes in plasma (Vilsboll et al., 2003). To bypass the potential short-lived effects of administration of the peptide (in contrast to physiologically produced and released endogenous GLP-1 which can be released for a longer time period), we used the synthetic GLP-1R agonist (Ex4) with considerably longer half-life. Ex4 is a compound originating from the saliva of the gila monster, a poisonous lizard which resides in New Mexico and Arizona. Endocrinologist Dr. John Eng discovered the peptide hormone in 1992 and was intrigued by its abilities to trigger insulin synthesis and release from the pancreatic β-cells (Eng et al., 1992). Ex4 shares 53% of sequence homology with human GLP-1; however Ex4 has an altered amino-acid in position two which renders the peptide resistant to the actions of DPP-IV (its primary metabolizer), giving it a half-life of several hours. In addition, GLP-1 itself was used in select experiments (Paper I). Selected doses were chosen based on their ability to reduce food intake and reward in previous studies, without altering the general condition of the animal, e.g. reduced locomotor activity or malaise (Alhadeff et al., 2012; Dickson et al., 2012; Dossat et al., 2011). In Paper I, 0.05 and 0.1 μg of Ex4 were used for intra-NTS injections in initial experiments; since the lower dose was sufficient to alter ingestive behavior the lower concentration was used for the remainder of the experiments. This dose was adapted from (Alhadeff et al., 2012). A dose of 0.1, 0.3 or 1 μg was used in Paper II for intra-PBN injections; the effects of Ex4 on this nucleus were previously unexplored, therefore several doses were used initially before finding an appropriate concentration. Paper III targeted the lateral ventricle, where 0.1 or 0.3 μg of Ex4 were applied; doses modified from (Dickson et al., 2012). Lastly, in Paper IV, 0.05 or 0.15 μg of Ex4 was applied to the LH.

EXENDIN-3(9-39)

To investigate the effects of endogenously acting GLP-1 we used a highly selective GLP-1R antagonist: exendin-3(9-39; Ex9), another compound discovered in the venom of the gila monster (Thorens et al., 1993). A dose of 20 μg of Ex9 was administered to the PBN in Paper II and 10 μg of Ex9 to the LH; doses modified from (Hayes et al., 2009).

ICI 182, 780 (ICI)

ICI is a highly selective estrogen receptor antagonist (Howell et al., 2000; Wakeling et al., 1991). It is used in the clinic as a treatment for certain types of breast cancer. A dose of 10 μg was injected into the lateral ventricle in Paper III; dose adapted from (Rivera and Eckel, 2010).

MPP DIHYDROCHLORIDE (MPPd)

MPPd is a highly selective ERα antagonist; it has a >200-fold selectivity for ERα over ERβ (Sun et al., 2002). Intraventricular injection of 1.4 μg was utilized in Paper III; dose adapted from (Martinez de Morentin et al., 2014).

All drugs were diluted in artificial CSF (aCSF), with the exception of ICI and MPP which were dissolved in 10 and 20% dimethyl sulfoxide (DMSO; a powerful dissolvent), respectively. The use of DMSO is not ideal; it has been suggested in numerous studies to produce toxic and/or adverse effects *in vivo*. However, it has been demonstrated that small volumes, of up to 75% DMSO, do not produce any aversive affects, or affect feeding behavior, after CNS injection (Blevins et al., 2002). In addition, aCSF with 10 or 20% DMSO was used as vehicle in these specific experiments, to reduce the risk of mistaking any potential effects on food reward, on the solvent.

EXPERIMENTAL PROCEDURES

CANNULA IMPLANTATION

To investigate the effects of a drug on the CNS, or within a specific brain area, guide cannulas were implanted in the area of interest through stereotaxic surgery. Briefly, rats were anesthetized using a ketamine/xylazine mix (3:1); the volume of the mix was administered as x = 1.5 x body weight in kg for females and 2 x body weight in kg for males. The rodents were then fixed to the stereotaxic frame. Guide cannulas (26 gauge; Plastics One, Roanoke, VA) were inserted into the area of interest using coordinates derived from a rat brain atlas (Paxinos & Watson). The following coordinates, presented as anterior/posterior (AP) to bregma, medial/lateral (ML) to midline and dorsal/ventral (DV) to skull, were used for each selected brain area:

- Lateral ventricle (Paper I, II and III): AP -0.9 from bregma, ML ±1.6 from midline and DV -2.0 (Paper I and III)/ -2.5 (Paper II) mm from skull, with injector aimed -4.0/4.5 mm ventral to skull.
- NTS (Paper I): AP on occipital suture/-4.9mm from bregma, ML ±0.75 from midline and DV -4.9 mm from skull, with injector aimed 6.9 mm ventral to skull.
- Lateral PBN (lPBN; Paper II): AP -9.5, ML ±2.0 from midline and DV -4.5 mm from skull, with injector aimed 6.5 mm ventral to skull.
- LH (Paper IV): AP -2.8, ML ±1.5 from midline, DV -, and -6.8 mm from the skull, with injector aimed 8.8 mm ventral to skull.

After being positioned according to the coordinates, the cannulas were fixed to the skull using jeweler's screws and dental cement, and closed with an obturator.

PLACEMENT CONFIRMATION

LATERAL VENTRICLE – ANGIOTENSIN II DRINKING TEST

Angiotensin II is a powerful stimulator of thirst; it induces immediate water intake (even in well-hydrated rats) when injected into certain brain regions, e.g. the third or lateral ventricle (Fitzsimons, 1976, 1998). In Paper I, II and III 20 ng/2 μL of angiotensin II was injected into the lateral ventricle. Rats were required to make contact with their water bottle within 5 minutes, and drink a

minimum of 5 mL of water within the 30 minutes following the injection for the cannula to be included in the study.

lPBN, LH AND NTS – HISTOLOGIC PLACEMENT CONFIRMATION USING INDIA INK

For cannulas targeting the lPBN, LH and NTS in Papers I, II and IV, cannula placement was verified *post mortem* by injecting India ink, in the same volume as for drug injections, into each area. After euthanization, the brains were collected, stored and sliced using a cryostat; placement was later examined using a microscope.

NTS – GLYCEMIC RESPONSE TEST

Initial verification of NTS cannula placement took place one week after stereotaxic surgery. Injection of 5-thio-D-glucose (5TG) into the caudal hindbrain causes hyperglycemia (Ritter et al., 1981). Sympathoadrenal-mediated glycemic response after microinjection of 5TG (24 μg/0.3μl) was therefore measured by collecting blood from the tip of the tail 30, 45, 50, 60, 70, 90 and 110 minutes after injection. An increase in blood glucose >100% increase was required for subject inclusion in the study.

VIRAL KNOCK DOWN OF GLP-1RS

To knock down the expression of GLP-1Rs in the LH in Paper IV, a short hairpin RNA (shRNA) targeting GLP-1R transcripts, developed by Schmidt *et al.* was used (Schmidt et al., 2016).

Following guide cannula implantation, the virus was injected bilaterally into the LH (0.5 μL per hemisphere). Injections were carried out using a micropump at a rate of 0.1 μL/minute (for 5 minutes); the injector was left in place for an additional 10 minutes to allow for diffusion away from the injection site. The knock down was later confirmed *post mortem* using RT-PCR.

BEHAVIORAL PROCEDURES

FOOD INTAKE AND BODY WEIGHT MEASUREMENTS

The effects of GLP-1 and its synthetic agonists on food intake and body weight are well known; however, which brain areas mediate these effects have not been fully investigated.

Paper I: food intake was measured 1 and 22 hours post injection; body weight change was measured at the 22 hour time point.

Paper II: the aim was to determine whether intra-PBN GLP-1R activation contributes to a reduced intake of different foods with varying caloric and palatable properties. The intake of chocolate pellets, liquid saccharine and standard chow was therefore measured at different time points after injection. Chocolate pellet consumption was measured 0.5, 1 and 2 hours after injection. Saccharine, a non-caloric sweetener, was used to measure the effects of GLP-1R stimulation in the PBN on palatability, without the influence of caloric content. Liquid saccharine intake was measured 4 hours after injection. To exclude the possibility that potential changes in intake are due to differences in food type (liquid vs solid), or potential effects on water homeostasis, water intake was also measured during this time period. Chow intake was measured 24 hours after injection. Body weight was measured immediately before and 24 hours after injection. For GLP-1R blockade experiments, food was removed 5 hours prior to injection (60 minutes before the dark cycle) to ensure equal levels of satiety. Chow intake was measured 1, 2, 3 and 24 hours after Ex9 injection. Body weight change was also measured at the 16-hour time point.

Paper III: Intake of chow and peanut butter was evaluated in non-deprived or overnight food-restricted rats. Peanut butter consumption was measured 1 hour after injection, and chow was offered for the remainder of the 24 hour time period. This experimental scheme was also used to investigate the effects of GLP-1R blockade in the PBN. The latter experiment was however only carried out in food restricted rats.

Paper IV: Chow intake was measured 1 and 24 hours after Ex4 or Ex9 injection. Prior to Ex4 experiments, rats were restricted to 50% of their normal intake overnight. For Ex9 experiments, rats were fasted overnight, and then given a 20-minute pre-meal of chow for 20 minutes before injection. This experimental design was modified from (Hayes et al., 2009) to elicit endogenous

GLP-1 release to a controlled meal. For GLP-1R knock down studies, food intake and body weight were measured daily for the entire duration of the experiment.

PALATABLE FOOD CHOICE TEST

To investigate if GLP-1R stimulation affects the intake of specific food types we utilized the palatable food choice test in Paper I. Preference for a palatable food (peanut butter) or chow was determined by offering the two foods simultaneously on the test day. In order to determine the effect of NTS GLP-1R activation on the palatable food choice, food presentation was preceded by intra-NTS microinjection of Ex4 or aCSF, and the amount of chow and peanut butter consumed was measured 1, 3 and 6 hours after injection. Variations in consumption due to neophobia, a fear of novel items, were reduced by familiarizing all the rats with peanut butter on at least one occasion prior to the test day.

PICA TEST

Nausea is one of the most common side-effects for individuals receiving synthetic GLP-1R agonist treatment (DeFronzo et al., 2005). Since rodents are not capable of emesis, the presence of nausea after treatment is measured using pica: the consumption of non-nutritive substances. Intake of kaolin, a form of white clay, was therefore measured in relation to food intake after Ex4 injection in Paper I. Rats were allowed to sample kaolin for at least 3 days before the Ex4 injection to avoid association of kaolin with Ex4 injections. Kaolin and chow intake were measured at 1, 3, 6 and 24 hours after injection in mildly food-restricted rats (10g of chow available overnight).

OPERANT CONDITIONING

Through operant conditioning, an individual makes an association between a particular behavior and a consequence, for example conducting a behavior to receive a reward (e.g. a sucrose pellet or electrical stimulation in a central reward area) or a punishment (e.g. foot shock). In regard to food reward, operant conditioning is commonly used to investigate the "wanting" component of food reward, i.e. the motivation to obtain a rewarding food.

To investigate the rewarding effects of palatable foods, rats in this study learned to press a lever in order to obtain a sucrose pellet (45 mg). The method was initially developed by Burrhus Frederic Skinner (Skinner, 1938), who developed a special box, the Skinner box, to study food-motivated behavior (*Figure 5*). The Skinner box, or operant conditioning box, main components include a signal light, one or two levers (one active), and a pellet dispenser.

Figure 5. Rodent using a Skinner box, a tool to measure operant conditioning behavior. This box is equipped with a signal light and one active lever. Boxes used in the present studies included two levers, whereas only one was active, indicated by a lit signal light. Boxes used in these studies also include a pellet dispenser and food tray which allows the rat to obtain a pellet in response to pressing the active lever.

The rats were trained and tested in operant conditioning boxes from Med-Associates. Training was conducted in four stages; rats were first trained on the fixed ratio 1 (FR1) schedule (a single press on the active lever results in the delivery of one sucrose pellet), followed by FR3 and FR5 (3 and 5 presses per pellet respectively). A minimum of 30 (Paper III and IV) or 50 (Paper I) responses per session on the active lever was required for the advancement to the next schedule. Training culminates with progressive ratio conditioning, where the number of presses on the active lever required to obtain one pellet increases exponentially according to the following equation: Pellets obtained = $5e^{0.2 \times \text{lever presses}} - 5$. Progressive ratio training is carried out until stable responding was achieved (number of pellets earned per session did not vary more than 15% for three consecutive sessions). All operant response testing was performed after the responses stabilized. The duration of FR sessions is 30 minutes and progressive ratio 60 minutes.

CONDITIONED PLACE PREFERENCE (CPP)

CPP is a learned behavior that occurs when a subject comes to prefer one place more than another due to the association of the given location with previously rewarding events. The CPP test is used to investigate the reinforcing effects of natural and pharmacological stimuli, including drugs of addiction. In this context a palatable food choice is paired to one of the chambers prior to injection of Ex4 or control.

The CPP test was performed in rats using an apparatus that is comprised of two connected chambers with distinct visual and tactile qualities (Med-Associates). CPP testing is carried out in three phases: habituation, conditioning and preference testing. During the habituation phase the preference for either chamber was assessed over the course of two days (20 minutes/day). The least preferred compartment (the compartment in which each rat spent the least amount of time during the two pretest days) was determined. Subsequently the least preferred compartment was paired with 4g of palatable food (chocolate or peanut butter). The conditioning phase consisted of 16 days of conditioning sessions (2 sessions per day). One day following the last conditioning session the rats received an intra-NTS Ex4 or aCSF injection, 30 minutes before CPP testing. The rats had free access to both compartments during testing, which took place in absence of the palatable food. The behavior of the animals was detected by infrared beams in each chamber and time spent in each compartment was determined. Time spent in each compartment reflects how rewarding the animal finds the substance/food that was paired to that specific compartment, and if the drug (Ex4) affects the rewarding properties of the palatable food.

To assure that all palatable food was consumed during the training sessions the rats had restricted access to chow in their home-cages (70% of normal daily chow intake) throughout the CPP experiment.

BIOCHEMICAL PROCEDURES

IMMUNOHISTOCHEMISTRY (IHC)

IHC is a microscopy-based technique for visualizing cellular components, such as proteins or other macromolecules, in tissue samples. It utilizes the specificity of antibodies binding to an antigen.

Antibodies, or immunoglobulins (Ig), are a large Y-shaped proteins synthesized by B-cells. There are five classes of antibodies: IgA, IgD, IgE, IgG and IgM, where IgG is most commonly used in immunohistochemistry. Antibodies are typically composed of two large heavy chains, and two small light chains. The two tips of the Y-shaped structure contain the antigen-binding sites which only bind to a specific antigen, in a lock and key manner. The tail, called the Fc fragment, has one binding site, which permits binding of other antibodies; an essential component in multistep immunohistochemistry (Ramos-Vara, 2005).

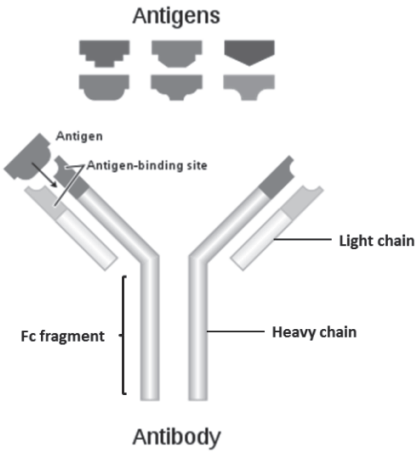

Figure 6. Representative image of the antibody structure. The antibody consists of two light chains and two heavy chains. The tips of the Y-shape contain an antigen-binding site which binds to a specific antigen in a lock-and-key manner. The antibody also has an Fc-fragment, which is critical for binding of other antibodies. Image modified from Wikimedia commons.

Antibodies used in immunohistochemistry are made by immunizing animals with the antigen of interest. The animal's immune system then responds by producing specific antibodies directed toward the antigen. Two types of antibodies are produced: polyclonal and monoclonal. Polyclonal antibodies bind to multiple sites on the antigen, but also to other epitopes depending on the

previous immune experience of the animal; therefore only a small fraction of the produced antibodies may actually bind to the actual site of interest on the targeted antigen. To minimize the risk of unspecific binding the collected antibodies therefore undergo affinity purification. Monoclonal antibodies are yielded by directly collecting B-cells from the spleen of an immunized animal. The B-cells producing only the antibody of interest are then fused with myeloma cells, carcinogenic plasma cells; the B-cell line therefore becomes immortal and divides continuously. Since these cells only produce the antibody of interest, monoclonal antibodies are therefore considered more specific than polyclonal; however they have lower sensitivity since they only bind to a single site of the antigen.

The antigen-antibody compound is visualized using a reporter molecule, often a fluorescent tag, which is bound directly or indirectly to the antibody. In this thesis, experiments utilized an indirect reporter molecule; staining therefore takes place through the following two steps: 1) Binding of an antibody (the primary antibody) specific to the antigen of interest, and 2) Binding of a secondary antibody to the primary; the secondary antibody also contains a fluorescent tag, which allows for detection of the antigen-antibody complex using a microscope (*Figure 7*).

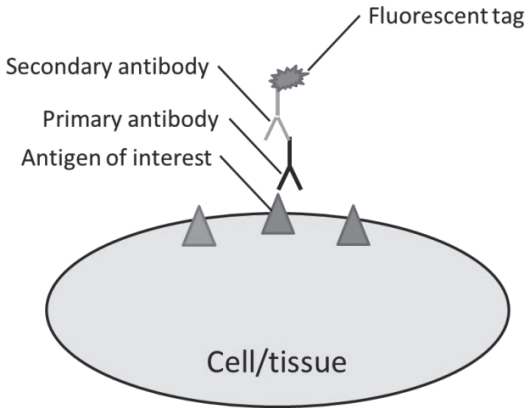

Figure 7. Illustration of the immunohistochemistry method using a labelled secondary antibody. The primary antibody binds to the antigen of interest. In a second step, a secondary antibody, attached to a fluorescent tag, binds to the primary antibody. The antigen-antibody complex can then be visualized using a microscope.

GENERAL

In Papers I and II, we utilized YFP-PPG mice for central GLP-1 detection; these mice already co-express PPG (the GLP-1 precursor) and YFP (the fluorescent protein) and need no further staining for visualization. However, to be able to visualize the presence of GLP-1 in relation to other molecules, such as TH (Paper I) or CGRP (Paper II), collected tissues require the use of immunohistochemistry to view the other molecular cell types.

TISSUE PREPERATION

The mice were anaesthetized with ketamine/xylazine solution and perfused transcardially with heparinized saline followed by fresh fixative solution (paraformaldehyde (PFA, 4%) in 0.1 M phosphate buffer. Briefly, the peritoneal cavity was exposed and a cut was made, parallel to the spine, to reveal the abdominal wall. The abdominal wall was carefully opened and the diaphragm cut open to reveal the heart. A 25 gauge needle, attached to a peristaltic pump, was inserted into the left ventricle and clamped in place. Heparinized saline was pumped through until clear solution flowed from the rodent and was followed by PFA perfusion which continued 10 minutes following contractions. Solutions were administered with a flow rate of 25 rpm.

Following perfusion, the brains were collected, and cut into coronal 25 μm sections using a cryostat; sections were then collected into tubes containing tissue storage solution. The tissue storage solution consisted of 50 ml glycerin, 50 ml ethylene glycol and 100 ml 0.1 M phosphate buffer (pH 7.5). Sections were then stored until use at 4°C.

IHC STAINING

The sections were first washed (3 x 15 min) in TNT with Triton-X (0.1%) (Sigma-Aldrich St.Louis, MO, USA).

Paper I: For TH visualization, the sections were incubated for two days in TNB blocking solution (Perkin Elmer, Akron, Ohio, USA) with 1:2000 Goat polyclonal antibody to TH (ab6211, Abcam, Cambridge, UK). The sections were washed in TNT with Triton-X (0.1%) and incubated in TNB blocking solution with 1:1000 Donkey anti-goat Alexa Fluor 568 (ab36001, Abcam).

Paper II: For CGRP visualization, the sections were incubated for 2 days in TRIS NaCl Boehringer Milk Powder-buffer (TNB-buffer) blocking solution (PerkinElmer) with 1:2000 goat polyclonal antibody to CGRP (ab36001; Abcam). The sections were then washed in TNT with Triton X-100 (0.1%) and incubated in TNB blocking solution with 1:1000 Donkey antigoat Alexa Fluor 568 (ab36001; Abcam).

The cell nuclei were stained with DAPI (1:5000; Life Technologies). The sections were then washed in TNT (2 × 15 min), submerged in 0.1 M phosphate buffer (PB), and mounted on microscope slides (Superfrost Plus; Menzel) together with ProLong Gold Antifade (Life Technologies).

NEUROANATOMICAL TRACING

Traditional neuronal tracing methods rely on axonal transport; the transport of molecules or organelles through the axon. Axonal transport can either be anterograde (forward; from cell body to axon terminals) or retrograde (backwards; from axon terminals to cell body). Retrograde tracing is used to investigate neuronal connections between a specific neuronal population and their inputs through various areas of the CNS.

Cholera Toxin Subunit B (CTB) is a nontoxic fragment from the cholera toxin secreted by the Vibrio cholera bacteria. Bacterial toxins have a high affinity to bind to specific sugars; after binding, the toxins are internalized by active transport via the nerve terminals and transported within the dendrites and axon to the cell body in the anterograde direction (Shehab et al., 2003). CTB binds specifically to the pentasaccharide chain of monosialotetrahexosyl ganglioside (its receptor), which makes it highly sensitive with minimal diffusion from labelled neurons (Lencer and Tsai, 2003).

TRACING OF LH-VTA PROJECTING NEURONS

Rats were anesthetized with a mix of ketamine, xylazine and acepromazine. A stereotax was used to target the VTA using the following coordinates: AP -5.0 mm, ML +1.0 mm and DV -7.6 based on zeroing for DV at the skull at -0.9 AP and +4.8 ML. Fluorescently labeled CTB (CTB-488, Invitrogen C22841) was injected using a micro-infusion pump (Harvard Apparatus) connected to a 33-gauge microsyringe injector attached to a PE20 catheter and Hamilton syringe. The compound was delivered with a flow rate of 5 µl/minute, and the injector was left in place 2 minutes after injection to allow for diffusion away from the injector before removal. Rats were sutured and allowed to recover and incubate with the virus for 10 days prior to sacrifice.

TISSUE PREPARATION

Rats were anesthetized and sedated with a mix of ketamine, xylazine and acepromazine; they were then transcardially perfused with 0.9% sterile saline (pH 7.4), followed by 4% paraformaldehyde in 0.1 M borate buffer (pH 9.5). Brains were collected and stored for later processing; they were then coronally

sliced in 20 μm sections using a cryostat, placed in a 0.02 M KPBS solution and mounted on slides which were placed into a vacuum chamber overnight.

Sections were postfixed in 4% paraformaldehyde in 0.1 M borate buffer (pH 7.4) for 1.75 hours, followed by 5 washes in KPBS. The sections were then pretreated by incubating them at 37 °C in pretreatment buffer (100 mM Tris buffer and 50 mM EDTA in distilled deionized water, pH 8) with 0.001% Proteinase K (Sigma P2308) for 30 minutes. This was followed by a 3 minute wash in incubation buffer and a 3 minute rinse in 100 mM Triethanolamine in water (pH 8). Sections were then incubated with 0.25% acetic anhydride in 100 mM triethanolamine for 10 minutes at room temperature followed by 2x2 minute washes in saline-sodium citrate buffer (1% citric acid trisodium/2% sodium chloride in water (pH 7.0)). Slides were then dehydrated in ethanol solutions in the following sequence: 50%, 70%, 95%, 100%, for 3 minutes/concentration and air-dried prior to hybridization.

FLUORESCENT IN SITU HYBRIDIZATION (FISH)

FISH is a technique used to localize specific segments of nucleic acid within a histologic section. It is based on the pairing of a specific sequence of nucleic acids with a complementary strand of DNA, which can be detected by attaching a reporter molecule (e.g. a fluorophore). This method is therefore distinct from IHC, since it detects a specific RNA or DNA sequence in a tissue, while IHC localizes proteins within a tissue.

VISUALIZATION OF GLP-1R EXPRESSING NEURONS

A hydrophobic barrier was drawn around each section and 3-4 drops of a probe targeting GLP-1R mRNA (GLP-1R, Advanced Cell Diagnostics 315221) was placed on each tissue section. The slides were incubated with the probe at 40 °C for 3 hours in a HybEz oven (Advanced Cell Diagnostics). Sections were washed with wash buffer (RNAscope®, Advanced Cell Diagnostics 320058) for 2 minutes. Reagents from RNAscope® Fluorescent Multiplex Detection Reagent Kit (Advanced Cell Diagnostics, 320851) were applied in order to amplify the probe signal: AMP1 was applied for 45 minutes, AMP2 for 30 minutes, AMP 3 for 45 minutes, and AMP4 for 30 minutes. Incubation steps occurred at 40 °C and each amplification step was followed by a 2 minute wash. Slides were mounted using ProLong® Gold Antifade mounting medium (Cell Signaling, 9071S) and a coverslip was placed over the sections.

CONFOCAL MICROSCOPY

Confocal microscopy is used to visualize a single plane of a tissue, increasing the optical resolution of the image; it is an important tool for determining potential co-localization between two or more molecular structures. By eliminating areas situated on top or below of the area of interest, it is possible to visualize, for example, if two proteins are expressed within the same neuron.

Focusing on one plane, or a thin optical section, is enabled by a pinhole placed in front of the detector; the pinhole allows the light to be focused in the specific plane only, within a range of only a few μm. Laser lights are then emitted separately at different wavelengths to excite the different fluorescent tags. Wavelengths used are within the fluorophores excitatory range, but that do not overlap with each other, to avoid leakage in between channels.

For IHC, fluorophore Alexa Fluor 568 (ab36001, Abcam) was used in both Paper I and Paper II for visualization of TH and CGRP respectively. Alexa Fluor 568 is an orange/red-fluorescent dye; excited by the 568 nm laser. YFP, the yellow/green fluorescent tag expressed in PPG cells in YFP-PPG mice, can be excited within a range of 490 to 510 nanometers; a lower wavelength within the span was chosen to avoid the risk of signal leaking from another fluorophore (for example Alexa Fluor 568). Diamidino-2-phenylindole (DAPI) is a blue fluorescent DNA stain which enables visualization of the cell nucleus, and is excited using a 405 nm laser.

Triple channel confocal images were acquired from 25 μm sections by alternating between lasers of different wavelengths. High resolution images were taken at 20x and tile scans were taken of areas of interest: NTS (three levels: caudal, area postrema and 4th ventricle; Paper I), and lateral and medial PBN (Paper II).

EPIFLUORESCENT MICROSCOPY

A fluorescent microscope is a traditional optical microscope equipped with a high-intensity light source, which enables the excitation of light of a specific wavelength to illuminate a sample.

The sample is illuminated with light of a specific wavelength, which excites the fluorophore. The illumination light is separated using an excitation filter, allowing for only the excited light to be transmitted and detected by the detector, providing a high signal- to- noise ratio.

Photomicrographs were acquired using a Nikon 80i (Nikon DS-QI1, 1280x1024 resolution, 1.45 megapixel) under epifluorescent illumination, or as optical slices using a Zeiss LSM 700 UGRB Confocal System (controlled by Zeiss Zen software). For details on confocal microscopy, see section above.

GENE EXPRESSION ANALYSIS

Gene expression is the process in which a gene from the DNA is transcribed and translated into a protein. Gene expression analysis is used to gain insight in the level at which a specific gene is expressed within a tissue; analysis can take place at several different steps in the expression process, during transcriptional, post-transcriptional, translational, and post-translational protein modification.

Transcription is the process in which a complementary RNA strand is made from the DNA sequence. This process is regulated by the binding of regulatory proteins, or through direct influence on the transcriptional machinery of the cell. The RNA strand is then spliced, in which noncoding intron sequences are removed, and the sequence is capped with a poly(A) tail, creating messenger RNA or mRNA. The mRNA is then transported from the cell nucleus to the cytosol where it can be translated to a protein, and undergo posttranslational modifications such as folding.

Gene expression can, for example, be analyzed by measuring mRNA levels using quantitative reverse transcription PCR (RT-qPCR). In this method, mRNA is first extracted and transcribed to a complementary DNA (cDNA) strand by reverse transcriptase. The cDNA template that is created is then amplified in the quantitative step, the qPCR reaction, in which fluorescence is emitted from commercially labeled hybridization probes. Using the standard curve, one can then determine the absolute number of copies of original mRNA.

RNA EXTRACTION AND RT-qPCR METHODOLOGY

Individual brain samples were homogenized in Qiazol (Qiagen, Hilden, Germany) using a TissueLyzer (Qiagen). Total RNA was extracted using RNeasy Lipid Tissue Mini Kit (Qiagen) with additional DNAse treatment (Qiagen). RNA quality and quantity were assessed by spectrophotometric measurements (Nanodrop 1000, NanoDrop Technologies, USA). For cDNA synthesis, iScript cDNA Synthesis kit (BioRad) was used. TaqMan PCR was used to quantify gene expression. The TaqMan gene expression assay uses a pair of unlabeled PCR primers which contains a probe attached to a fluorescent dye label on the 5´ end, and a non-fluorescent quencher at the 3´ end. When the dye and quencher are in close proximity of each other, the dye is quenched and

cannot fluoresce. When the cDNA segment binds to the primer and the probe, the probe is cleaved by TaqMan polymerase and the fluorescent dye is released from the quencher, enabling florescence. The amount of fluorescence is relative to the level of the gene expressed, which can be calculated using the ΔΔCt method (Livak and Schmittgen, 2001). Peptidylprolyl isomerase A (PPIA) or β-Actin were used as reference genes. The selection of the specific reference gene was based on the assessment of their stable expression between control and test samples.

DETERMINATION OF ESTROUS CYCLE PHASE

As mentioned above, the rodent estrous cycle is a 4-5 day cycle which consists of 4 phases: diestrus, proestrus, estrus and metaestrus. The diestrus phase is characterized by the presence of neutrophils, also known as leukocytes, which appear as small, round cells with multiobulated nuclei. Proestrus samples consist mainly of small nucleated epithelial cells. These cells are small, albeit larger than neutrophils, and possess a round or oval shape. They are non-keratinized, with a high nuclear to cytoplasmic ratio, and often stain darker than neutrophil cells. Estrus mainly consists of large cornified, nucleated epithelial cells; these cells have a lower nuclear to cytoplasmic ratio and are much larger than the other cell types. The estrus phase also includes large anucleated keratinized epitheal cells, which are large, aged cells that lack nuclei. Lastly, metestrus is characterized by the presence of all cell types, though neutrophils are in the majority. Each cycle phase varies in its average duration, with proestrus lasting approximately 14 hours, estrus 24-48 hours, metestrus 6-8 hours and diestrus 48-72 hours in rats (B., 1939; Long J. A., 1922; M., 1951).

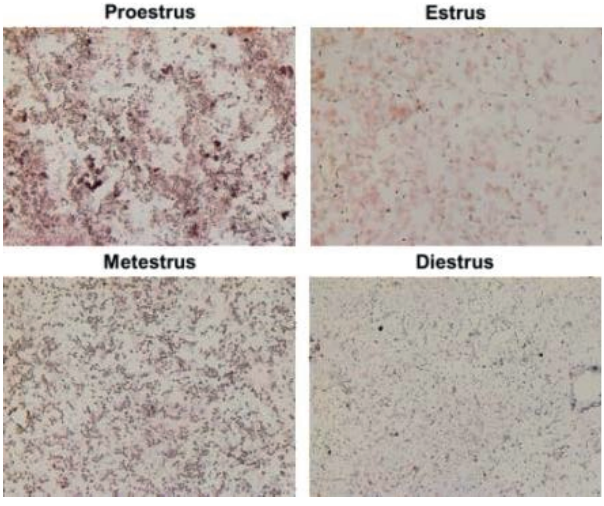

Figure 8. Representative images of each phase of the estrous cycle: proestrus, estrus, metestrus (diestrus I) and diestrus (diestrus II).

METHODOLOGY

Smears were collected by injecting 50 μL of saline into the opening of the vagina with a pipette, and flushing 2-3 times before sucking up the liquid and ejecting it on a glass slide. Estrous cycle phase was initially assessed by microscope examination of unstained smear preparations collected from the females each morning, or immediately after operant conditioning. Cycle phase was additionally, later confirmed after Papanicolaou staining as described previously (Chateau et al., 1996). Briefly, vaginal samples were stained with Hematoxylin and Eosin using the following protocol: Samples were incubated in Hematoxylin for 10 minutes, rinsed in distillated H_2O and placed in eosin for 2 minutes. They were again rinsed with H_2O prior to dehydration using 70% ethanol for 1 minute, followed by 5 minutes in 95% ethanol. Finally, the slides were placed in xylene for 10 minutes and allowed to dry before slides were mounted with Pertex glue and covered with a coverslip. The samples were observed using a standard, white light microscope and cycle phase was determined.

STATISTICAL ANALYSIS

All the data are presented as mean ± Standard Error of the Mean (SEM). Statistical significance was analyzed using Student's t-test, or one- or two-way analysis of variance (ANOVA) when appropriate (GraphPad Software, Inc., San Diego, CA).

The student's t-test was used for comparisons between treatment groups; the unpaired t-test was used when comparison took place between two separate groups while paired t-test was conducted when values were compared within the same individuals, e.g. in counterbalanced experiments. ANOVA was used for comparisons between two or more treatment groups, when comparing one or more independent variables. ANOVA can be carried out using one-way or two-way analysis: one-way analysis is used when comparing one independent variable (e.g. treatment), while two-way analysis is used for two independent variables (e.g. treatment and sex).

p-values lower than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

PAPER I

GLP-1 reduces food intake by acting on GLP-1Rs located in key central energy balance regulating areas, such as the hypothalamus and the brainstem (Barrera et al., 2009; Goke et al., 1995; Hayes et al., 2009; Hayes et al., 2008; McMahon and Wellman, 1997, 1998; Pannacciulli et al., 2007; Turton et al., 1996). In addition, GLP-1 has been shown to reduce food reward behavior by acting on receptors within the mesolimbic system (Alhadeff et al., 2012; Dickson et al., 2012; Dossat et al., 2011). Previous data therefore suggest a neuroanatomical segregation between the homeostatic and hedonic effects of the peptide. In Paper I, we aimed to challenge this view and hypothesized that GLP-1 can regulate food reward behavior by acting directly on the brainstem, on GLP-1Rs in the NTS, the primary source of central GLP-1.

NTS GLP-1R ACTIVATION PREFERENTIALLY AFFECTS THE INTAKE OF PALATABLE FOOD

To determine if the actions of GLP-1 on food intake in the NTS are affected by the palatability of the food item, we conducted a palatable food-choice test. Direct NTS GLP-1R stimulation was shown to specifically reduce the intake of the palatable food item (peanut butter), without affecting the intake of chow, the non-palatable food choice (chow; *Figure 9A*). Thus, GLP-1's actions on food intake seem to be selective to palatable foods.

Chemical lesions of the area postrema and NTS were previously shown to exclusively reduce the intake of palatable food, but not chow, suggesting a selective role for the NTS in food intake regulation (Ritter and Edwards, 1984; South and Ritter, 1983). Since GLP-1R signaling in the current study seems to function in the same manner, it is interesting to postulate that NTS GLP-1 neurons may represent at least one of the neuronal populations lesioned in these studies.

When chow was offered as the sole food choice, Ex4 significantly reduced chow intake (*Figure 9B & C*). In addition body weight was reduced 22 hours after treatment (*Figure 9D*). These results are in line with previous studies by *Hayes et al* demonstrating the anorexic actions of GLP-1R stimulation within this area (Hayes et al., 2009; Hayes et al., 2008).

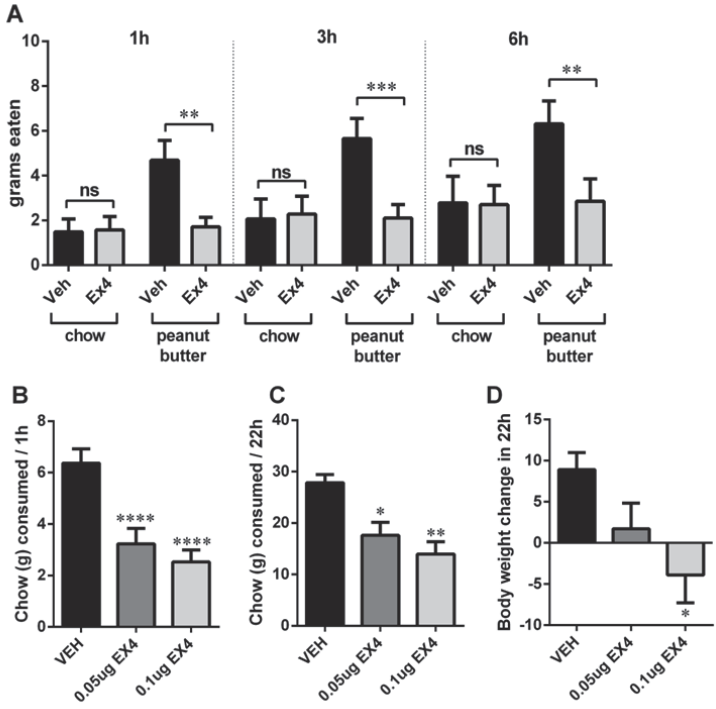

*Figure 9. Direct NTS GLP-1R stimulation with Ex4 (0.05 μg) suppressed the intake of palatable food (peanut butter), but not chow, when both food types were offered simultaneously (A). However, when chow is offered alone, GLP-1R stimulation significantly reduces the intake of chow 1 (B) and 22 hours (C) after injection. A significant reduction in body weight was also observed at 22 hours (D). Data are expressed as mean ±SEM. n = 12 per treatment group (A) and n = 11 per each treatment group (B-D). * p<0.05, ** p<0.01, *** p<0.005.*

The anorexigenic actions of GLP-1R stimulation in the NTS were not associated with nausea (*Figure 10*). This is an important factor since nausea is one of the primary side-effects of synthetic GLP-1R agonist treatment in humans, which often leads to discontinuation of the treatment (DeFronzo et al., 2005). While food intake and body weight were significantly reduced (*Figure 10A*) after direct microinjection of Ex4 into the NTS, there was no effect on the intake of kaolin (*Figure 10B*), suggesting that the nausea-inducing effects of GLP-1R treatment are mediated through other central GLP-1R populations, and not through actions on GLP-1R in the NTS.

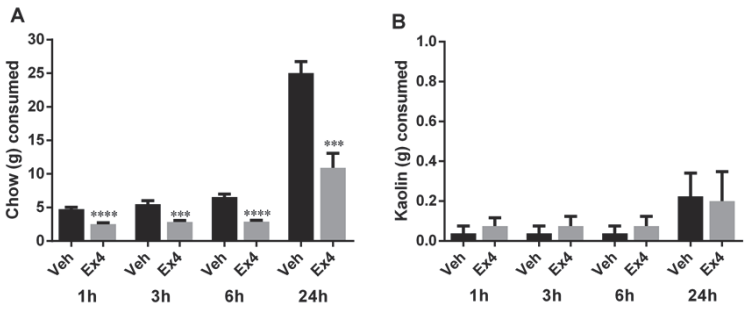

*Figure 10. GLP-1R stimulation in the NTS reduced chow intake (A), without altering the intake of the nonnutritive food item kaolin (B). Data are expressed as mean ±SEM. n = 8 per treatment group. ***p< 0.005, **** p<0.0005.*

ACTIVATION OF GLP-1RS IN THE NTS REDUCES FOOD REWARD BEHAVIOR

In addition to the anorexigenic effects on intra-NTS GLP-1Rs, GLP-1R stimulation in this area also reduced food-motivated behavior (*Figure 11)*. Ex4 injection, or injection of the endogenous peptide GLP-1, into the NTS, significantly reduced the amount of sucrose rewards earned (*Figure 11A & D*) and lever presses for sucrose (*Figure 11B & E*) in the operant conditioning test. Importantly, there was no effect on locomotor activity (*Figure 11C & F*), indicating that the effects of Ex4 and GLP-1 on motivated behavior in this area are not due to motor impairment or malaise.

GLP-1R stimulation in the NTS also reduced food-reward behavior in the CPP test (*Figure 12)*; Ex4 injected rats spent significantly less time in the food-paired chamber compared to the vehicle-treated group (*Figure 12A*). Ex4 treated rats also displayed a preference shift toward the non-food paired chamber (*Figure 12B*).

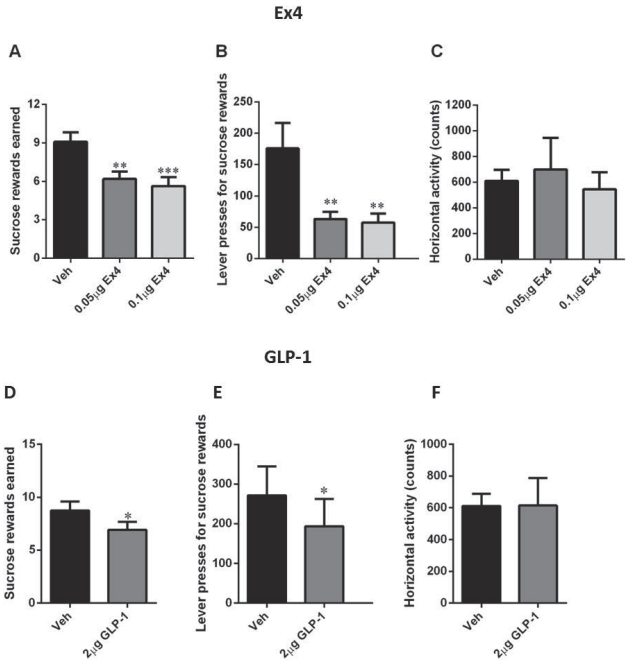

*Figure 11. GLP-1R stimulation by synthetic agonist Ex4, or GLP-1, potently reduced the number of sucrose rewards earned (A & D) and active lever presses (B & E) in the operant conditioning test. Importantly, this suppression in food-motivated behavior was not associated with a reduction in locomotor activity (C & F). n =11 per each treatment group. * p<0.05, ** p<0.01, ***p < 0.0005.*

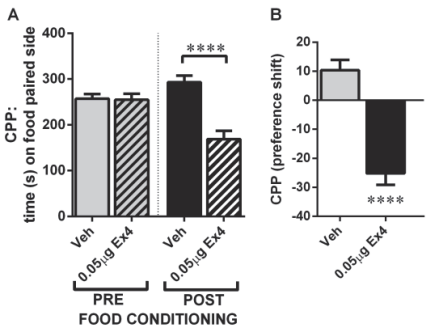

*Figure 12. Intra-NTS Ex4 administration reduced the preference for the food-paired chamber (A) and shifted the preference to the non-food paired chamber (B) in the conditioned place preference (CPP) test. The preference (%CPP) was calculated using the following formula: ((test-pretest)/ (total time-pretest))x100. Data represent mean ±SEM. n=11 (vehicle group) and 8 (Ex4 group). **** p<0.0005.*

GLP-1 FIBERS ARE IN CLOSE PROXIMITY TO NTS NORADRENERGIC NEURONS

The demonstrated effects of GLP-1R stimulation in the NTS may be mediated by NTS GLP-1R activity transmitted to the mesolimbic system. NTS neurons were previously shown to project to both the NAc and the VTA; these neurons (A2) express TH, the precursor for adrenaline, noradrenaline and dopamine, and are a major source of noradrenaline to the NAc (Delfs et al., 1998; Rinaman, 2011; Wang et al., 1992). Interestingly, NTS A2 neurons have already been implicated in the control of rewarding effects of substances of abuse (Delfs et al., 2000; Olson et al., 2006). Based on these previous results we decided to investigate if GLP-1 neurons possess the ability to interact with NTS A2 neurons, providing a potential connection to the reward system.

Taking advantage of the venus mice which express PPG-YFP we confirmed the distribution of YFP-labeled cell bodies compared to previous studies. The location of TH positive cells is also in line with the neuroanatomical position of NAc-projecting TH neurons previously reported *(Figure 13)* (Larsen et al., 1997; Llewellyn-Smith et al., 2013; Rinaman, 2011; Wang et al., 1992)

In addition, TH immunoreactive neurons in the NTS were found to receive close appositions from PPG-YFP fibers, indicating that GLP-1 may exert its effects on reward by acting on mesolimbic-projecting A2 neurons. This interaction was observed at the level of the caudal NTS (*Figure 13A & B*) and at the level of the area postrema (*Figure 13C & D*). At more rostral levels of the NTS, by the caudal 4th ventricle, very few PPG-YFP fibers were identified (*Figure 13E & F*).

In addition to NTS noradrenergic A2 neurons, TH immunoreactivity may also mark adrenergic C2 neurons, which can also be found in the NTS (Minson et al., 1990; Rinaman, 2011). However, the presence of C2 neurons is relatively sparse in the areas of the NTS exhibiting GLP-1 fiber detection, therefore most of the TH positive cells visualized in this study are most likely noradrenergic.

EX4 INJECTION IN THE NTS INCREASES TH AND DOPAMINE 2 RECEPTOR EXPRESSION IN THE VTA

To further evaluate if GLP-1R stimulation in the NTS affects areas of the mesolimbic system, gene expression of dopamine-related genes, in addition to other genes previously associated with changes in reward behavior, was investigated in the NAc and VTA after Ex4 injection. Intra-NTS Ex4

administration led to a significant increase in TH and dopamine 2 receptor mRNA expression in the VTA (*Figure 14A*).

TH expression was previously shown to increase in response to GLP-1R stimulation in the VTA (Mietlicki-Baase et al., 2013), suggesting that GLP-1R stimulation in the NTS and the VTA may lead to similar effects on dopamine production. In addition to the NAc, dopaminergic VTA neurons also project to the amygdala, and increased dopamine activity in this area has previously been associated with reduced sucrose-driven food-motivated behavior (Anderberg et al., 2014). Connections transferred from the NTS to the amygdala (NTS-VTA-Amygdala) therefore provide a potential mechanism for the reward-reducing effects of GLP-1 acting in the NTS.

In addition to TH, the expression of dopamine 2 receptors was also increased in the VTA in response to intra-NTS GLP-1R stimulation. Dopamine 2 receptors in the VTA are primarily thought to function as autoreceptors, mediating a negative feedback effect on the dopaminergic neurons on which they are expressed, resulting in a reduction in dopamine release. However, only select populations of dopaminergic neurons are inhibited by these receptors; dopaminergic neurons innervating the NAc are inhibited by dopamine 2 receptors, while nearly none of the amygdala projecting dopaminergic neurons are affected (Margolis et al., 2008). Therefore, it is possible that the reduction in food reward behavior after intra-NTS GLP-1R stimulation in this study is due to the inhibitory action of GLP-1 on dopamine neurons projecting to the NAc. mRNA levels of TH and dopamine 2 receptors were not altered in the NAc after intra-NTS Ex4 injections (*Figure 14C).*

The expression of other dopamine receptors was not altered in either the VTA or the NAc (*Figure 14A & C).* In addition, the expression of reward related genes: FBJ osteosarcoma viral oncogene B (FosB), transcription factor cAMP responsive element binding protein 1 (Creb1) and glutamate decarbolylase 1 (Gad1) were also measured. No changes in mRNA expression of these genes were detected after intra-NTS Ex4 administration, neither in the VTA or the NAc *(Figure 14B & D*).

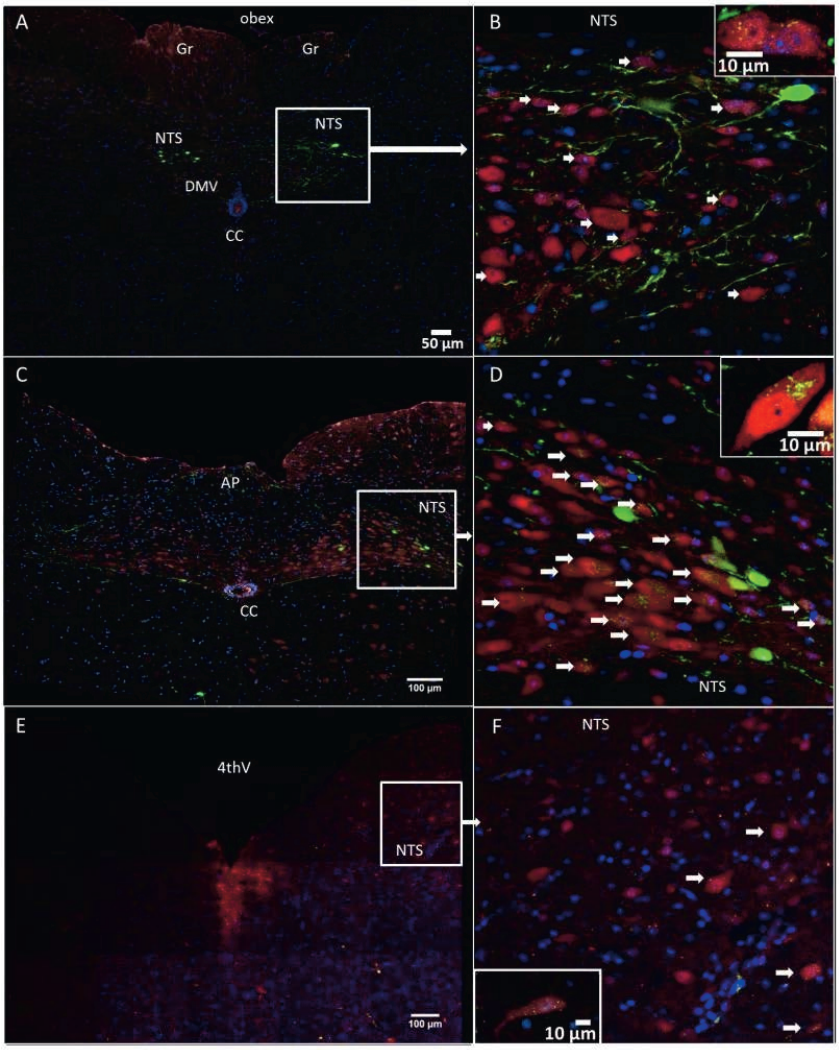

Figure 13. Yellow fluorescent protein (YFP)-immunoreactive axons (green) are in close apposition to tyrosine hydroxylase (TH) positive neurons (Egecioglu et al.) of the NTS. Micrographs show representative images of three levels of the NTS: the caudal NTS (A-B), the NTS at the level of the area postrema (C-D) and the NTS at the level of the 4th ventricle (E-F). White arrows indicate NTS tyrosine positive neuronal cell bodies closely apposed to GLP-1 fibers. NTS = nucleus of the solitary tract, AP = area postrema, cc = central canal, DMV = dorsal motor nucleus of the vagus, GR = gracile nucleus, 4thV = 4th ventricle.

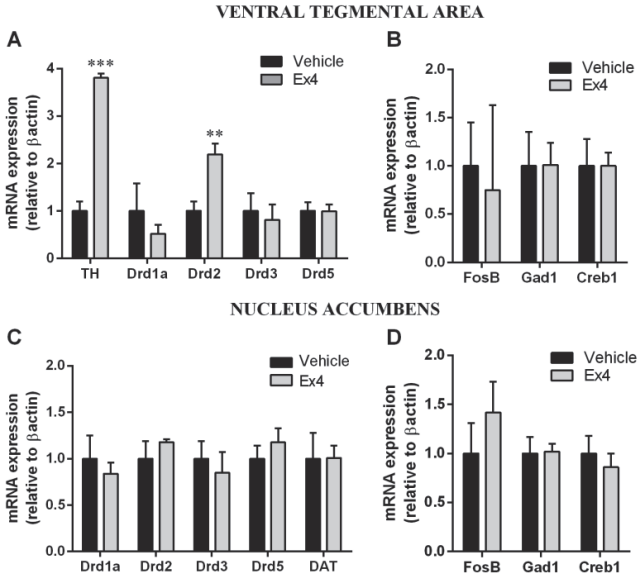

*Figure 14. Intra-NTS GLP-1R activation increased mRNA expression of the gene that encodes tyrosine hydroxylase (TH), and the dopamine 2 receptor (Drd2) without significantly altering mRNA expression of other dopamine receptors in the VTA (A). Expression of reward-related genes FosB, Creb1 and Gad1 remained unchanged (B). Receptor expression in the nucleus accumbens was unaltered (C & D). Data are expressed as mean ±SEM. n = 6 (vehicle group) and n = 5 (Ex4 group). ** p<0.01, *** p<0.005. Drd1a = Dopamine receptor 1, Drd3 = dopamine receptor 3, Drd5 = dopamine receptor 5, Gad1= glutamate decarboxylase 1, Creb1 = cAMP responsive element binding protein 1, FosB = FBJ osteosarcoma viral oncogene B.*

PAPER II

The PBN is an important nucleus in food intake regulation; it integrates signals from the hypothalamus and periphery, as well as the immune system, to control energy balance (de Araujo, 2009). Inhibitory hypothalamic projections to the PBN are critical for normal food intake maintenance, and loss of these signals leads to starvation (Carter et al., 2013; Wu et al., 2009; Wu and Palmiter, 2011; Wu et al., 2013). In addition, viscerosensory stimuli result in neuronal activation of the PBN (Wu et al., 2009; Wu et al., 2012; Wu et al., 2013). However, the origin and character of this neuronal input was recently unknown. The PBN expresses GLP-1Rs and GLP-1 mRNA has been found in this area. In Paper II we therefore investigated if GLP-1 neurons, originating from the NTS, project to the PBN and if GLP-1 acts in this area to regulate feeding behavior.

HINDBRAIN GLP-1 NEURONS INNERVATE THE PBN

Fibers from fluorescent YFP-PPG neurons, most likely originating from the NTS (*Figure 15A & B*), were detected in the medial and lPBN. Over half (55±1.2%) of the cell bodies in the lPBN were in close proximity to these fibers, while approximately 31% (31±2.3%) of the cell bodies in the medial PBN appeared to be innervated (*Figure 15C-F*).

These data are in line with previous studies showing that the PBN is densely innervated by NTS neurons; in addition, innervation from the NTS was shown to overlap with GLP-1 positive terminals, and NTS-PBN projecting neurons were indicated to co-localize with NTS GLP-1 producing neurons (Alhadeff et al., 2014; Rinaman, 2010).

GLP-1R STIMULATION IN THE lPBN REDUCES FOOD INTAKE AND BODY WEIGHT

Intra-PBN injection of Ex4 significantly reduced the intake of chocolate pellets (*Figure 16A*), liquid saccharine (*Figure 16B*) and chow (*Figure 16C*). In addition, 24-hour body weight change was significantly reduced (*Figure 16D*).

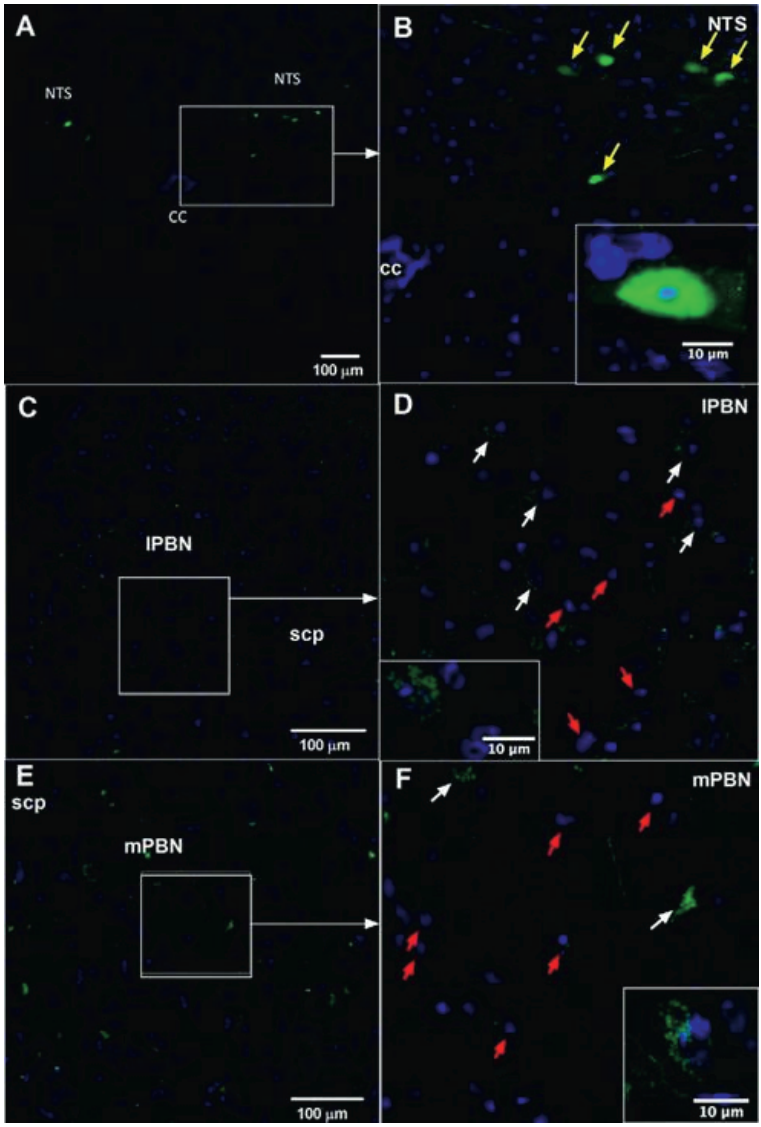

Figure 15. Fluorescent YFP-PPG neurons (green) and DAPI (nuclear stain, blue) in coronal sections throughout the NTS and the PBN of YFP-PPG mice. Micrographs showing the cell bodies of green YFP-immunoreactive PPG neurons (yellow arrows) in the NTS (A and B), micrographs showing the lateral PBN (lPBN; C and D), and the region of the medial PBN (mPBN) just below the superior cerebellar peduncles (E and F). White arrows indicate PBN cell bodies in close apposition to GLP-1 fibers, whereas red arrows indicate cell bodies in this region that were not apposed by GLP-1 fibers. Insets in D and F show the interaction at a single cell level. cc, central canal, scp = superior cerebellar peduncles. B, D, and F show higher magnification of areas in A, C, and D, respectively.

These results demonstrate that GLP-1R stimulation in the PBN reduces the intake of foods with a variety of different caloric and palatable characteristics. Saccharine is a non-caloric sweetener, therefore a reduction in its intake suggests that GLP-1 modulates intake in this area regardless of caloric content. Importantly, the reduction is not due to differences in food type (liquid vs solid), or potential effects on water homeostasis, since water intake was not altered by Ex4 injection (*Figure 16B*).

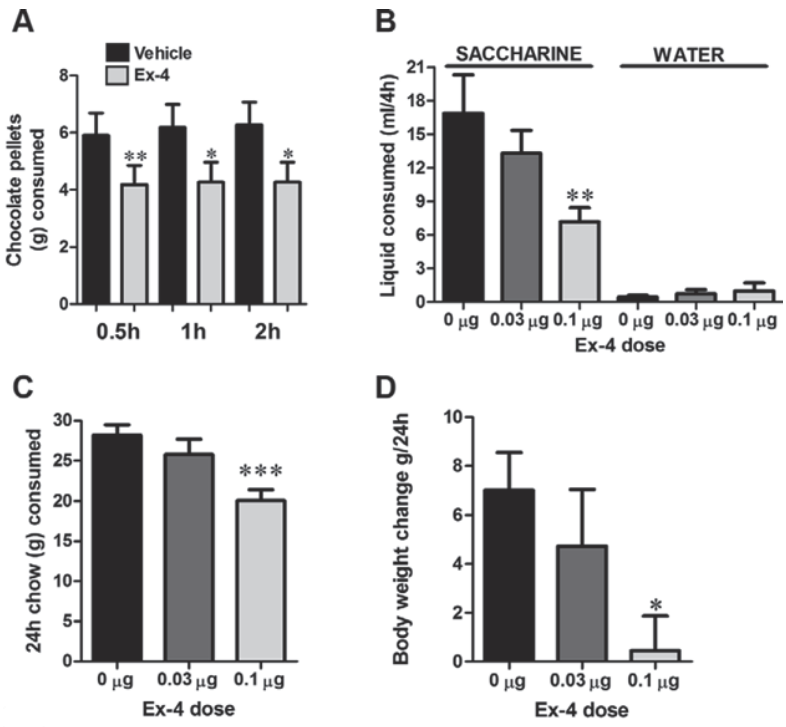

*Figure 16. Lateral PBN (lPBN) injection of Ex4 (0.1 μg) reduced 2-hour chocolate pellet consumption (A), in addition to reduced liquid saccharine consumption, but not water intake, during a 4 hour time period (B) Chow intake (C) and body weight (D) were also reduced 24 hours after injection. Data are expressed as mean ± SEM. n=11 (chocolate pellet group), n=12 (saccharine group) and n=11 (chow intake group). *p<0.05, **p<0 .01, ***p<0 .005.*

ENDOGENOUS GLP-1 REGULATES FEEDING AND BODY WEIGHT VIA PBN GLP-1RS

Blockade of GLP-1Rs in the lPBN, using the GLP-1R antagonist Ex9, significantly increased chow intake 2 and 3 hours after injection (*Figure 17A*). The effect of Ex9 on chow intake was no longer present after 16 hours (*Figure 17B*), but the manipulation did lead to a significant increase in overnight body weight change (*Figure 17C*). These data indicate that not only pharmacological stimulation of GLP-1Rs in this area, but also endogenously released GLP-1, acts in the lPBN to regulate food intake and body weight.

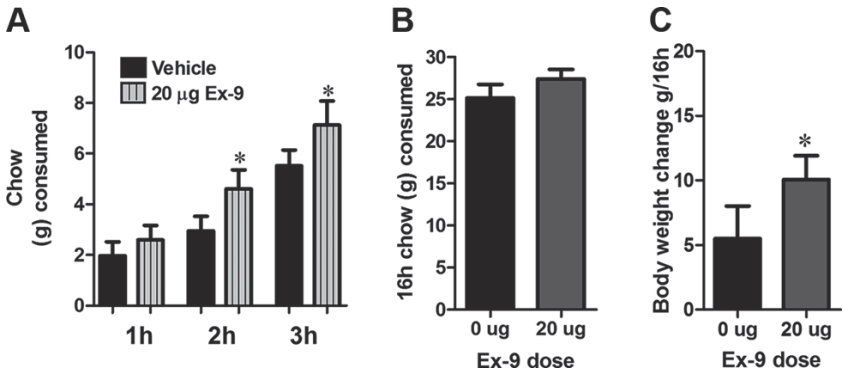

*Figure 17. Intra-PBN delivery of Ex9 increased chow intake 2 and 3 hours after injection (A). Overnight chow intake, measured 16 hours after Ex9 injection, was not altered (B). However, Ex9 increased 16-hour body weight gain. (C). Data are expressed as mean ± SEM. n = 12. *p<0 .05.*

CENTRAL GLP-1R ACTIVATION INDUCES INTERLEUKIN-6 (IL-6) AND CALCITONIN GENE-RELATED PEPTIDE (CGRP) EXPRESSION IN THE PBN

In order to begin to understand the potential downstream circuitry activated by GLP-1R stimulation in the PBN, gene expression of molecules recently shown to be associated with suppression of appetite in the PBN was analyzed (Carter et al., 2013; Roman et al., 2016; Shah et al., 2014; Wu et al., 2009).

Ex4 injection into the lateral ventricle led to a significant increase in the expression of the inflammatory signal IL-6 in the PBN of *ad libitum* fed and overnight-fasted rats; while interleukin-1β (IL-1β) expression was unaffected under both conditions (*Figure 18C & D*). GLP-1 mediated increase of IL-6 expression is in line with previous research; IL-1β and IL-6 were previously shown to mediate the appetite-suppressing effects of GLP-1 in the hypothalamus and brainstem (Shirazi et al., 2013a). In fact, in addition to their role in the inflammatory system, IL-1β and IL-6 have also been implicated to play a role in the regulation of metabolic function in healthy animals. In addition, IL-6 or IL-6 receptor knockout in mice leads to obesity and disturbed glucose metabolism (Erta et al., 2012; Garcia et al., 2006; McGillicuddy et al., 2011; Wallenius et al., 2002). Therefore, in addition to the hypothalamus and brainstem, IL-6 seems to mediate the effects of GLP-1 on food intake in the PBN.

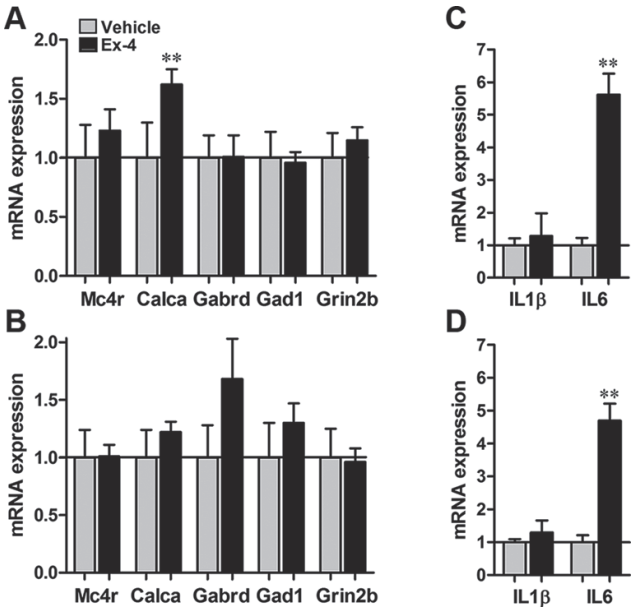

*Figure 18. GLP-1R activation increased mRNA expression of the gene that encodes CGRP (Calca), without significantly changing the mRNA expression of other genes previously shown to be associated with changes in food intake in the PBN in ad libitum fed rats (A). Gene expression in the PBN was unaltered after Ex4 injection in overnight-food restricted rats (B). Ex4 increased the expression of IL-6, but not IL-1β, in both ad libitum-fed (C) and fasted (D) rats. Data are expressed as mean ± SEM. n = 6-9. Mc4r = melanocortin 4 receptor, CGRP = calcitonin gene-related peptide, Gabrd = γ-Aminobutyric acid, Gad1 = Glutamate decarboxylase 1, Grin2b = glutamate ionotropic receptor NMDA type subunit 2B, IL1β = interleukin 1β, IL6 = interleukin 6. **p<0 .01.*

Central GLP-1R stimulation also led to a significant increase in the expression of CGRP in the PBN, in *ad libitum* fed rats (*Figure 18A*). However, this change was not seen in overnight-fasted rats (*Figure 18B*). CGRP neurons are exclusively found in the lPBN and have been shown to play an important role in appetite suppression (Carter et al., 2013; Lutz et al., 1997; Paues et al., 2001).While CGRP-neurons have been shown to reduce food intake regardless of feeding state, the ability of GLP-1 to reduce food intake has previously been shown to depend largely on nutritional status. GLP-1 reduces appetite in a fed, but not fasted state; this effect is thought to be based on the amount of GLP-1Rs available on the plasma membrane, with increasing levels of GLP-1Rs trafficked to the membrane in response to a meal (Ronveaux et al., 2014). Taken together, although stimulation of CGRP-expressing neurons have been shown to reduce food intake regardless of feeding state, animals that underwent an overnight fast may have had a reduced number of GLP-1Rs available on the cell membrane, and the level of activation of these cells may therefore be insufficient to further stimulate CGRP neurons in this area. Thus, neuronal activation of CGRP-expressing neurons could therefore be a potential mechanism in which GLP-1 can mediate its anorexic effects in the PBN.

lPBN CGRP NEURONS ARE INNERVATED BY GLP-1 FIBERS

In order to determine a potential connection between GLP-1 and CGRP-expressing neurons in the lPBN we examined if CGRP neurons in this area are innervated by GLP-1 fibers.

Fluorescent fibers from YFP-PPG neurons were found in close proximity to CGRP neurons (*Figure 19A & B*). In fact, most of the GLP-1 fibers found in the lPBN innervated CGRP-positive cells, and nearly half of the CGRP-expressing cells in the lPBN receive GLP-1 innervation (*Figure 19C & D*).

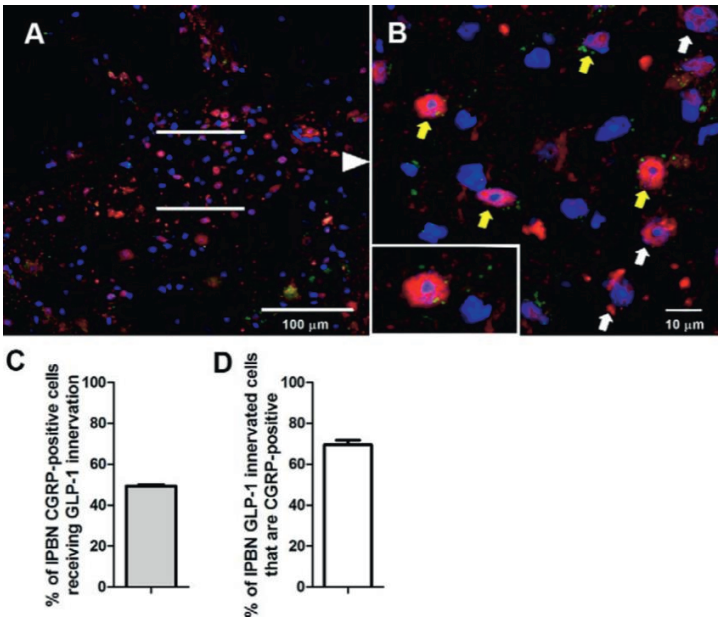

Figure 19. YFP-immunoreactive axons (green) were found in close apposition to CGRP neurons (red) in the lateral PBN (lPBN; A). Yellow arrows indicate CGRP-labeled lPBN cell bodies closely apposed by the GLP-1 fibers, whereas white arrows indicate CGRP-labeled cell bodies in this region that were not apposed to GLP-1 fibers. Higher magnification of the lPBN region (B).Cell nuclei are stained with DAPI (blue). Half of the CGRP-positive cells in the lPBN receive GLP-1 innervation (C), and the majority of GLP-1 innervated cells were CGRP-positive (D).

INTRA-PBN INJECTION OF CGRP REDUCES FOOD INTAKE

Direct injection of CGRP into the lPBN resulted in a significant reduction in food intake; however, the effect of CGRP on food intake was short-lived and only lasted approximately 2 hours (*Figure 20A*). No significant differences in food intake were seen 3 or 16 hours after treatment (*Figure 20A & B*). These results indicate that part of the effects of GLP-1 on food intake in the lPBN may be due, in part, to its actions on CGRP neurons and receptors in this area. To further determine the necessity of CGRP- neurons for the effects of intra-lPBN GLP-1R stimulation on food intake, further experiments blocking these neurons prior to intra-lPBN GLP-1R stimulation, together with food intake measurements, are required. However, in addition to CGRP-neuronal activation, other central mechanisms are most likely also involved, at least in the long term regulation of food intake of the peptide, as CGRP only produces a transient reduction on food intake, and isn't sufficient to produce long term changes in body weight.

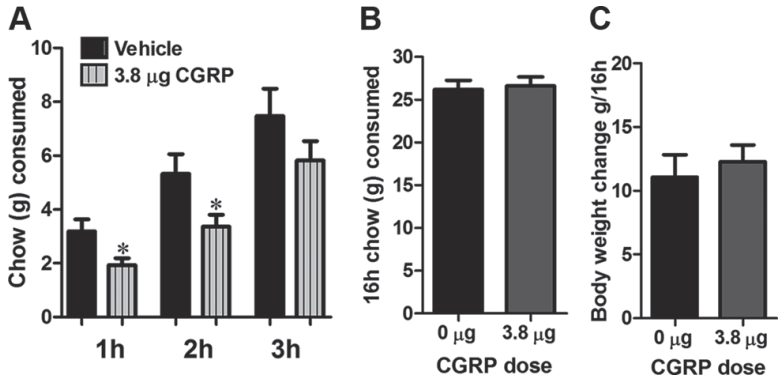

*Figure 20. Lateral PBN injection of CGRP reduced chow consumption up to 2 hours after injection (A). This effect was short lasting; chow intake (B) and body weight (C) measured 16 hours after injection were not altered. Data are expressed as mean ± SEM. n = 12.*p<0 .05.*

PAPER III

The effects of certain food-intake regulating hormones, for example leptin and ghrelin, have been shown to have differential effects in males and females (Clegg et al., 2006; Clegg et al., 2007; Clegg et al., 2003). In addition, sex differences have been implicated in drug addiction, modulating the rewarding effects of drugs of abuse by acting in reward-regulating areas (Becker, 1990; Becker et al., 1984; Justice and De Wit, 2000; Zhang et al., 2008). However, little is known about the actions of sex hormones on food reward. In this paper we aimed to investigate potential differences, between sexes, in the effects of GLP-1 on food intake and reward.

SEX MODIFIES THE EFFECT OF CENTRAL GLP-1R STIMULATION ON FOOD REWARD

To investigate potential differences in the central effects of GLP-1R stimulation on food-motivated behavior, a group of rats of each sex received intracerebroventricular injections of Ex4 prior to operant-conditioning testing.

Central GLP-1R stimulation reduced food-motivated behavior in females, but not males, in *ad libitum* fed rats (*Figure 21A & B*). While the main effect of sex on these parameters was not significant, there was a significant interaction between the effect of Ex4 and sex. After overnight food restriction, central Ex4 treatment successfully reduced food-motivated behavior in both sexes, although to a larger extent in females than males (*Figure 21E & F*). The interaction between drug treatment and sex was also significant, indicating a sex difference in the central effects of GLP-1R stimulation on food reward.

The effects of Ex4 treatment on food intake did however not differ between sexes. While there was a significant reduction in both palatable (peanut butter) and standard food intake after Ex4 treatment, the effects of the treatment on males and females were not significantly different (*Figure 21C, D, G & H*). These results suggest that the sex differences in food-reward behavior may be specific to the "wanting" component of food reward, and not "liking". However, one must take into account that peanut butter consumption is not an ideal test for palatability, or "liking", especially in comparisons between sexes, since one cannot rule out differences in ingestion, satiety levels or an interaction with satiety signals. "Liking" is commonly investigated by studying facial expressions in response to a taste, where positive, or "liking", responses include licking of the lips and rhythmic tongue protrusions (Berridge et al., 2009). These tests were not included in the current study; examination of these behaviors is

necessary to draw more accurate conclusions regarding the effects of Ex4 on palatability.

Despite previous studies demonstrating a reduction in food-reward behavior in males after Ex4 treatment (Dickson et al., 2012; Shirazi et al., 2013b), males in this study did not exhibit any effects of treatment on food reward in the non-food deprived state. The absence of this effect may be due to differences in feeding state during training and testing, as rats in the specified studies were food restricted for these tasks, while rats in the current experiment were not; this may indicate a sensitivity to feeding state in the effects of Ex4 on food-motivated behavior.

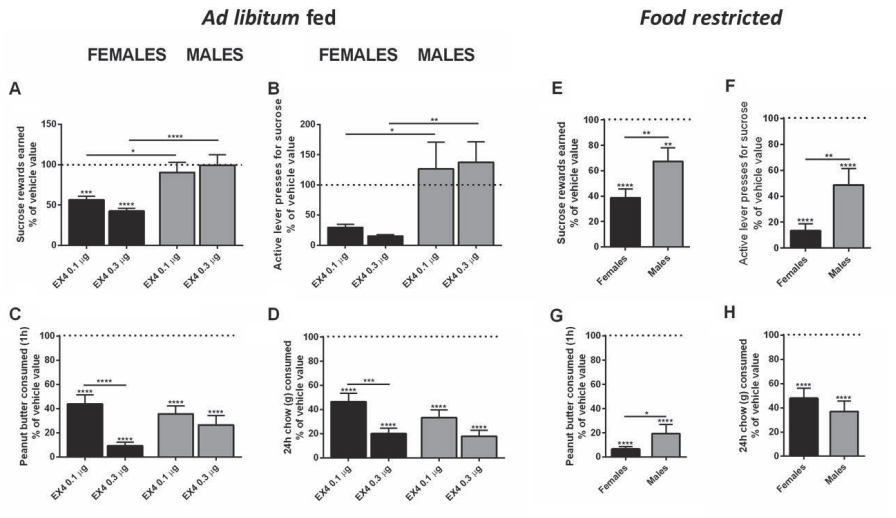

*Figure 21. Central Ex4 treatment led to a significant reduction in sucrose rewards earned (% of vehicle value; A) and active lever presses for sucrose (% of vehicle value; B) in ad libitum fed females, but not males. In the food restricted feeding state, Ex4 treatment led to a significant reduction in both parameters in both sexes (E & F). Intake of peanut butter and chow were significantly reduced in both ad libitum fed and food restricted males and females; however, the effect of treatment did not differ between sexes (C, D, G, H). Data are expressed as mean ± SEM. n = 20 (females) and 18 (males) per treatment group in the ad libitum condition and n = 10 (females) and 9 (males) per treatment group under the food restricted condition. *p < 0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

CENTRAL ESTROGEN BLOCKADE ATTENUATES THE EFFECTS OF EX4 ON FOOD-MOTIVATED BEHAVIOR

To investigate if the sex differences in the effects of GLP-1R stimulation on food-motivated behavior are mediated by estrogens, central estrogen receptors (ERα and ERβ) were blocked prior to central Ex4 injections and operant conditioning testing in food-restricted males and females. Central estrogen receptor blockade, using the selective estrogen receptor antagonist ICI, led to the attenuation of Ex4's effects of on food-motivated behavior in females (*Figure 22C & D*). Interestingly, similar effects of estrogen receptor blockade on this behavior were also present in males, since the effects of Ex4 on sucrose rewards earned were attenuated by pretreatment with ICI (*Figure 22A*). Ex4 treatment in males did not result in a significant reduction in active lever presses for sucrose (*Figure 22B*). Male and female data combined revealed a significant difference between the Ex4 vs ICI/Ex4 groups in sucrose rewards earned (*Figure 22E*). Importantly, ICI treatment alone did not have any effects on reward-related behavior.

In contrast to food-motivated behavior, estrogen receptor blockade did not attenuate the effects of Ex4 on the intake of palatable food (peanut butter) in males or females (Figure 23A & C), nor did it attenuate the effects of Ex4 on 24-hour chow intake in males (Figure 23B). Surprisingly, Ex4 did not significantly reduce peanut butter in males in the current experiment, though it has been shown to do so in previous studies (Dickson et al., 2012). This difference may be due to an insufficient amount of test subjects. In contrast to peanut butter, ICI treatment attenuated the Ex4-mediated reduction in chow intake in females (*Figure 23D*).

Since estrogen is mainly considered a "female hormone", it may be surprising that the hormone is able to increase the potency of Ex4 in both sexes. However, estrogen is produced in the periphery and brain in both sexes (Marrocco and McEwen, 2016). In addition, estrogen has previously been shown to potentiate the effects of Ex4 in both sexes in one recent studies where co-administration of GLP-1 and estrogen in a conjugated form was shown to reduce food intake and body weight, at doses of the hormones that were ineffective alone, in both males and females (Finan et al., 2012; Vogel et al., 2016). Taken together with the current data, this suggests that estrogen signaling is sufficient to enhance the effects of centrally acting GLP-1 on food-motivated behavior in both sexes, but not necessary for its effects on food intake in males.

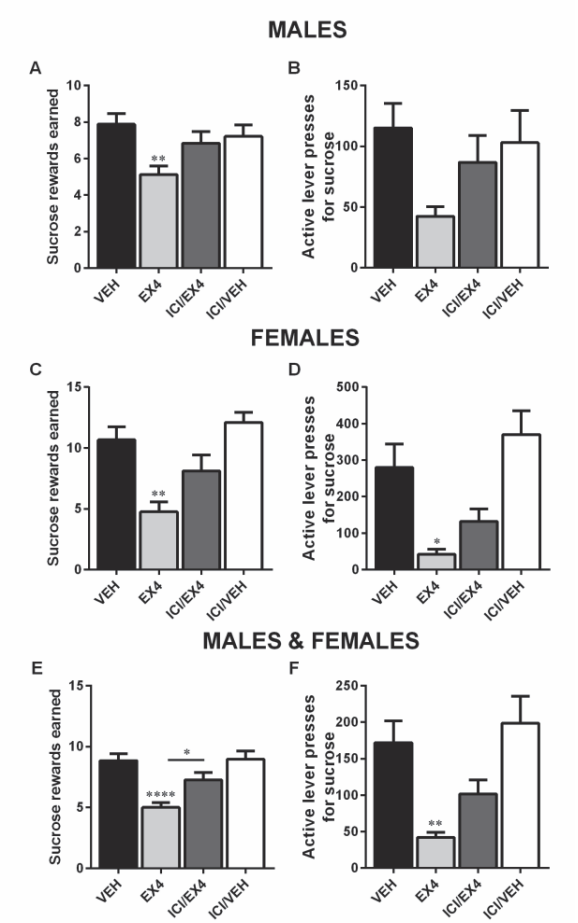

*Figure 22. Combined treatment with the selective estrogen antagonist ICI and Ex4 attenuated the reduction in sucrose rewards earned in females (C) and males (A) compared to Ex4 treatment alone; this effect was however not significant. Reduction of active lever presses for sucrose was also attenuated in females after ICI treatment (D). Active lever presses was not significantly reduced by Ex4 treatment in males (B). The number of sucrose rewards earned for males and females combined was reduced after central Ex4 injection (E); this effect was attenuated after co-administration with ICI. Combined results for males and females also demonstrate a significant reduction in lever presses for sucrose after central GLP-1R stimulation (F); attenuation by the ICI treatment did not reach statistical significance. Data are expressed as mean ± SEM. n = 10 (females) and 18 (males) per treatment group. *p<0.05, **p<0.01, ****p<0.0001.*

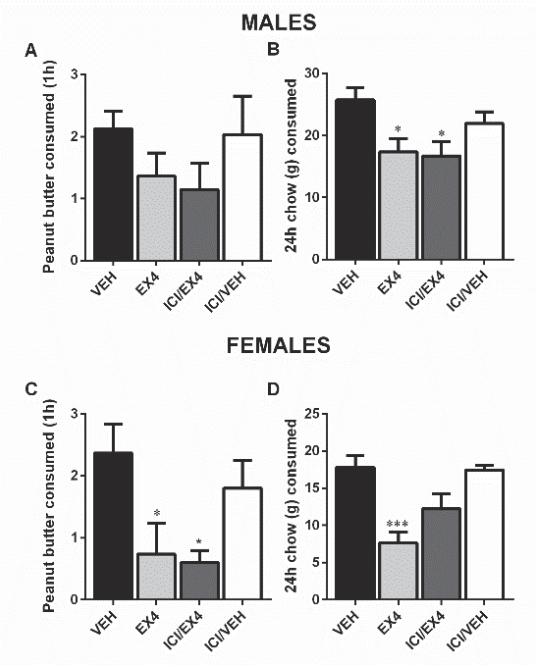

*Figure 23. Central GLP-1R stimulation did not reduce peanut butter intake in males (A); it did however reduce the intake of peanut butter in females (C), and chow in both sexes (B & D). ICI only attenuated the actions of Ex4 on chow intake in females (D). Data are expressed as mean ± SEM. n = 10 (females) and 9 (males) per treatment group for peanut butter measurement and n = 10 (females) and 18 (males) per treatment group for chow intake. *p<0.05, ***p<0.001.*

THE ESTROGEN-MEDIATED POTENTIATION OF EX4'S EFFECTS ON FOOD-MOTIVATED BEHAVIOR MAY BE DUE TO ACTIONS ON ERα

ERα has previously been shown to play an important role in homeostatic food intake and body weight regulation, while studies have failed to find a connection in these behaviors with ERβ (Asarian and Geary, 2013). In addition, ERβ, but not ERα, activation was previously shown to increase cocaine-seeking behavior (Larson and Carroll, 2007), in contrast to potentiating a reduction in food-reward in the current study; we therefore aimed to investigate if the enhanced effects of estrogen on GLP-1R activation induced effects on food intake and reward were mediated by ERα.

ERα blockade using the specific ERα antagonist MPPd led to an attenuation of the Ex4-mediated reduction in sucrose rewards earned in both males and females (*Figure 24A and C*), and active lever presses for sucrose in females (*Figure 24D*). In males, Ex4 treatment did not lead to a significant reduction in active lever presses for sucrose (*Figure 24B*). When analyzing males and females combined, there was a significant difference between the Ex4 and MPPd/Ex4 injected groups (*Figure 24E*). While the effects of Ex4 on active lever presses for sucrose of males and females was attenuated by MPPd treatment, differences between the Ex4 and MPPd/Ex4 groups did not reach significance (*Figure 24F*). In contrast, MPPd treatment did not attenuate the effects of Ex4 on peanut butter or chow intake in either sex (*Figure 25A-D*).

While the neural systems controlling ingestive and motivated behaviors are connected, food also has the ability to tap into, and affect, one of these systems without altering the other. For instance, peripheral signals transmitting signals about food intake and energy balance can increase intake in times of metabolic need, while appetitive foods, such as those high in sugar and fat, can increase the drive to eat when no such need is present as previously discussed. Therefore, it is plausible that ERα only affects the "liking" and "wanting" aspects of Ex4s effects on food intake, without altering general consummatory behaviors.

ERα antagonist MPPd was previously shown to be insufficient to attenuate the effects of estradiol on food intake (Santollo and Eckel, 2009). MPPd did however reduce the estrogen-related decrease in food intake present in the estrus phase of intact, cycling females; it may therefore be of interest to analyze the effects of ERα blockade on Ex4 treatment in females, divided based on cycle phase, in future studies, as cycle phase was not determined in the present study.

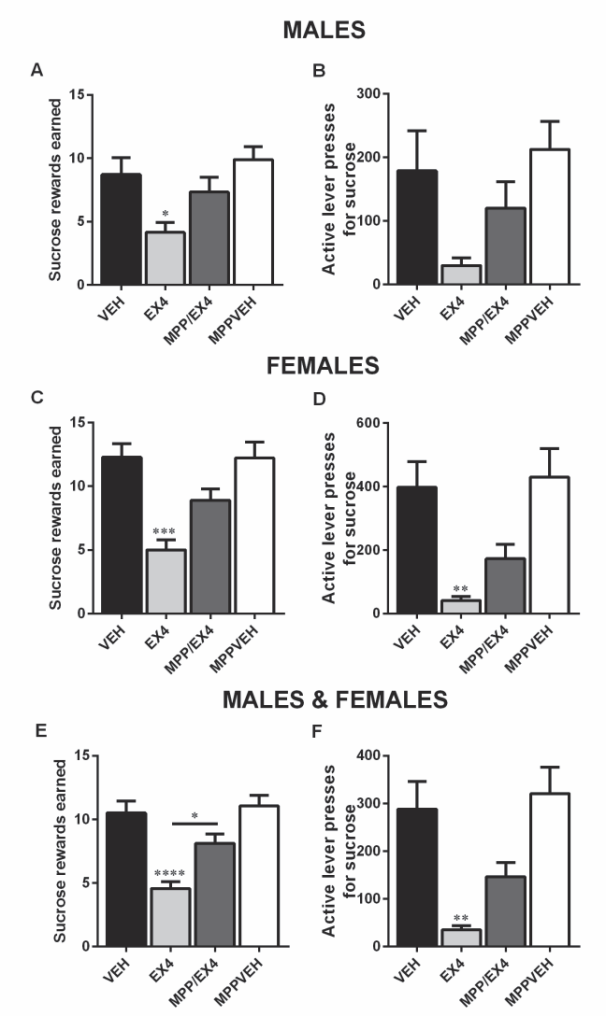

*Figure 24. Central administration of Ex4 in food-restricted rats led to a reduction in sucrose rewards earned in females (C) and males (A). Ex4 no longer significantly reduced sucrose rewards earned after administration of the specific ERa-antagonist MPPd. Ex4 treatment also reduced lever presses for sucrose earned in females (D) and produced a trend for a reduction in males (B). Compilation of female and male data revealed a significant reduction in sucrose rewards earned (E) and active lever presses (F) after Ex4 treatment that was attenuated after combined MPP/Ex4 treatment. Data are expressed as mean ± SEM. n = 7–9 for males and females. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

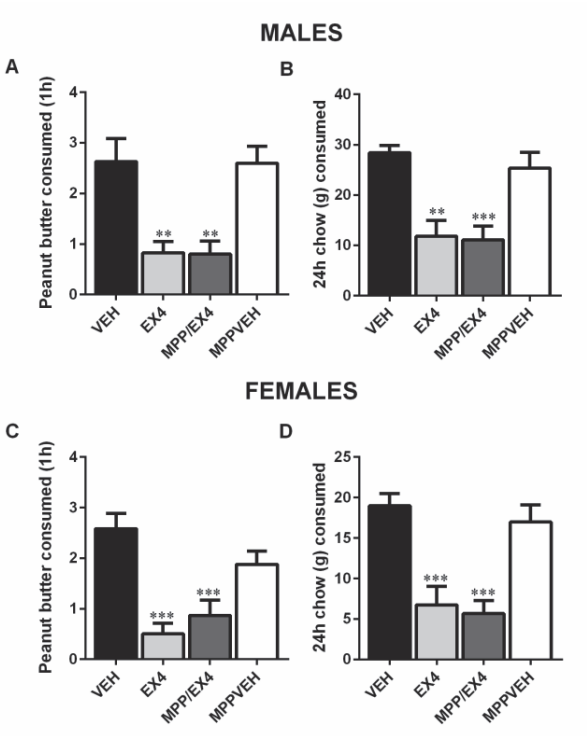

*Figure 25. Central injection with the GLP-1R agonist Ex4 significantly reduced peanut butter intake and chow intake in males (A & B) and females (C & D). The reduction was still present after treatment with the ERa-antagonist MPPd. Data are expressed as mean ± SEM. n = 7–9 for males and females. **p<0.01, ***p<0.001.*

PAPER IV

The LH is known as "the feeding center" and has been shown to play an important role in the drive to feed. In addition, the nucleus also regulates motivated behavior and is one of the most potent self-stimulation centers of the brain (Hoebel and Teitelbaum, 1962; Margules and Olds, 1962; Olds, 1962); the LH is therefore suggested to act as an interface between the homeostatic and hedonic control of feeding behavior, integrating metabolic and reward signals. GLP-1 neurons innervate the LH and GLP-1Rs are expressed throughout this area (Larsen et al., 1997; Merchenthaler et al., 1999); however, despite studies demonatrating the role of GLP-1 in the regulation of food reward in other areas, whether this peptide alters food-reward behavior by acting in the LH was previously unknown.

GLP-1RS ARE PRESENT ON LH-VTA PROJECTING NEURONS

To investigate a potential connection between GLP-1 in the LH and the reward system, we began by examining if LH-VTA projecting neurons express GLP-1Rs.

Approximately 55% of LH-VTA projecting cells contain GLP-1R mRNA, suggesting that the VTA may be one of the underlying targets behind potential effects of intra-LH GLP-1R stimulation on food reward behavior (*Figure 26*).

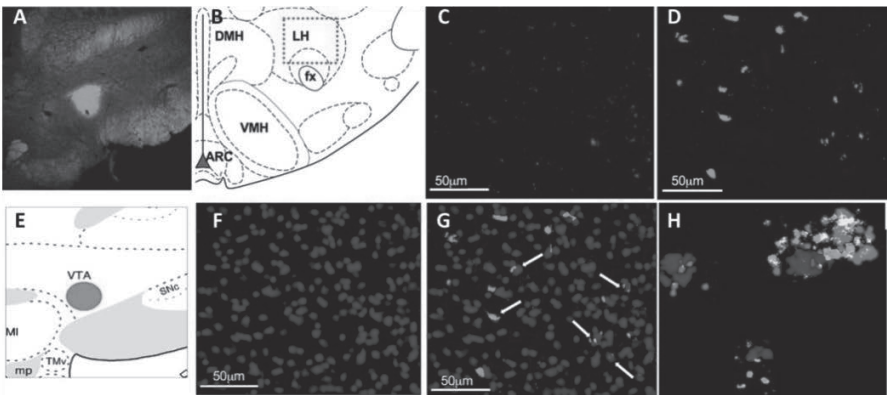

Figure 26. Representative images of GLP-1Rs on LH neurons that project to the VTA. Site of injection in the VTA of cholera toxin subunit B (CTB) conjugated to AlexaFluor 488 (A). Back-labeled cells were present in the rostral LH region, dorsal to the fornix (B). Representative images of GLP-1R in situ hybridization (red; C), back-labeled CTB-positive neurons from the VTA (green; D) and DAPI nuclear stain (blue; F). Merged image showing colocalization of GLP-1R and CTB in the LH (white arrows; G), and a confocal image of a double-labeled GLP-1R and CTB-positive neuron with DAPI counterstain (H).

ACUTE LH-TARGETTED GLP-1R ACTIVATION REDUCES FOOD INTAKE AND FOOD-MOTIVATED BEHAVIOR

After finding a potential connection with the reward system, we next investigated if LH GLP-1R stimulation indeed alters food-motivated behavior.

In male rats, intra-LH injection of Ex4 led to a significant reduction in sucrose-rewards earned (*Figure 27A*) and active lever presses for reward (*Figure 27B*). In addition, chow intake was reduced 1 and 24 hours after treatment (*Figure 27C & D*).

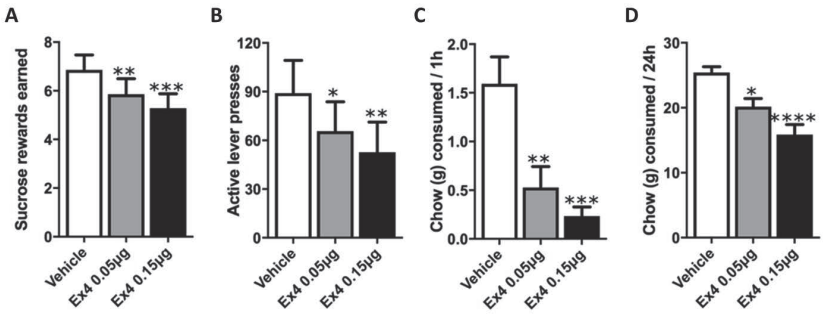

*Figure 27. Intra-LH GLP-1 analogue Ex4 microinjection reduced the amount of sucrose rewards earned (A,) and the number of lever presses for the rewards (B), in a progressive ratio schedule in male rats. Food ingestion is also affected, as illustrated by a potent reduction in 1 (C) and 24 hour (D) chow intake. Data are expressed as mean±SEM. n=14–15 (male rats). *p<0.05, **p<0.01,***p<0.001, ****p<0.0001.*

In addition, the same treatment produced a slightly less potent suppression in food-motivated behavior in females, where only the higher dose of Ex4 was sufficient to reduce sucrose rewards earned (*Figure 28A*); active lever pressing did not reach significance at any of the doses administered (*Figure 28B*). Food ingestion was also affected by intra-LH Ex4 treatment in females; however, 1 hour intake in females was only reduced after administration of the higher dose of Ex4, while both doses significantly reduced 24- hour chow intake (*Figure 28C & D*).

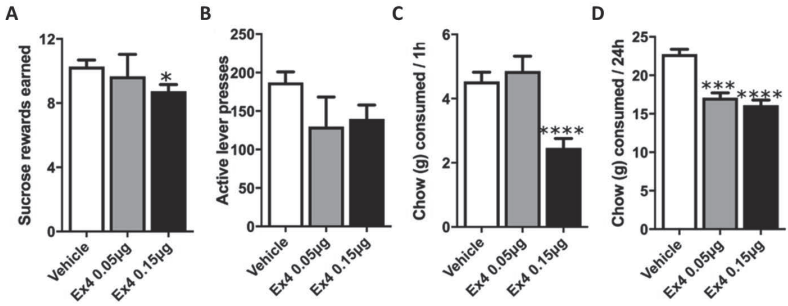

*Figure 28. In females, only the higher dose of Ex4 reduced the amount of sucrose rewards earned (A) and resulted in a trend to reduce the number of lever presses for rewards (B) in the operant conditioning test. Food ingestion was also affected in female rats as illustrated by a reduction in 1 (C) and 24 hour (D) chow intake. Data are expressed as mean ±SEM. n=9-37. *p<0.05, ***p<0.001, ****p<0.0001.*

EFFECTS OF INTRA-LH GLP-1R STIMULATION ON FOOD INTAKE AND FOOD-MOTIVATED BEHAVIOR IS DEPENDENT ON ESTROUS CYCLE PHASE

Estrogen levels vary with the reproductive cycle and these variations have been shown to affect appetite, both through actions on ERs and by interacting with various peptides, such as leptin, ghrelin and CCK. In addition, sex and estrogens have been shown to play a role in reward behavior, with direct effects of cycle phase on the reward system. Due to the sex differences observed in Ex4's effect on food-reward behavior in the previous experiment, we decided to investigate if these differences are due to an influence of the hormonal variations of the estrous cycle.

Females in the estrus, but not mestaestrus/diestrus, responded to intra-LH Ex4 treatment with reduced food-motivated behavior (*Figure 29A & B*). Food intake was significantly reduced in all examined estrous phases (*Figure 29C & D*).

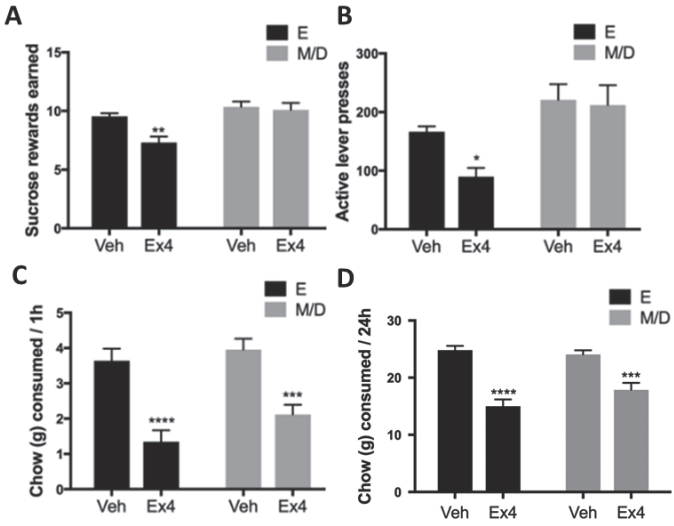

*Figure 29. Intra-LH Ex4 reduced the effort to obtain a reward (A & B) in a sucrose-motivated progressive ratio task in females in the estrus phase (E), but not metestrus or diestrus (MD) phases, of their cycle. Ex4 potently reduced ingestive behavior in all females irrespective of the cycle phase (C & D). Data are expressed as mean ±SEM. n=19 (vehicle in E), n=20 (Ex4 in E), n=8 (vehicle in MD) and n=8 (Ex4 in MD). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

LH GLP-1R ACTIVATION ALTERS LH NEUROPEPTIDE EXPRESSION IN A SEX DIVERGENT MANNER

To investigate potential downstream mechanisms of the effects of intra-LH GLP-1R stimulation, gene expression of molecules previously shown to alter food intake and reward in this area was examined (Reichelt et al., 2015; Schroeder and Leinninger, 2018; Shirazi et al., 2013a).

Intra-LH Ex4 treatment led to a significant increase in the anorexic interleukins IL-1 and IL-6 in males; in addition, MCH expression was reduced in this area (*Figure 30A*). In females, the estrous-dependent differences in the effect of Ex4 on intake and reward were mirrored by the gene expression results. Expression of IL-1, IL-6, neurotensin and orexin was reduced in females, only in the estrus phase (*Figure 30B*). A reduction in MCH was detected in all cycle phases. Changes in LH gene expression are similar between males and females in estrus, though males display a stronger IL-6 induction than females, while females in metaestrus/diestrus show no changes in IL-1 and IL-6 expression (statistical comparisons not shown).

Interleukins IL-1 and IL-6 have previously been suggested to mediate the anorexic effects of GLP-1 in the CNS (Shirazi et al., 2013a). In addition, the expression of MCH, the orexigenic LH neuropeptide, was reduced in response to LH GLP-1R stimulation in both sexes. The increase in anorexic interleukins, and reduction in orexigenic MCH expression, most likely contribute to the food intake and food-reward reducing effects of GLP-1R stimulation in the LH.

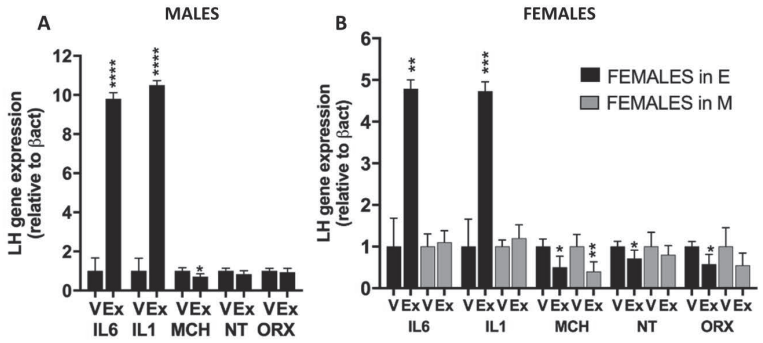

*Figure 30. Ex4 administration selectively within the LH increased the expression of interleukins IL-1 and IL-6 in males (A) and females in the estrus (E) phase, but not meteaestrus/diestrus (M) phases (B). In addition, melanin-concentrating hormone (MCH) expression was reduced in males (A), and in all females, regardless of cycle phase (B). The expression on neurotensin (NT) and orexin (ORX) was reduced in response to intra-LH Ex4 treatment in females. Data were normalized to the housekeeping gene β-actin and are expressed as mean ±SEM. n=19 (vehicle in E, n=20 (Ex4 in E), n=8 (vehicle in M) and n=8 (Ex4 in M). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

In females, there was also a significant reduction in orexin expression. A potential link between GLP-1 and orexin neurons in the LH was previously suggested by Acuna *et al*, where GLP-1 was shown to depolarize orexin neurons (Acuna-Goycolea and van den Pol, 2004); a reduction in neuronal activity of these neurons could therefore also contribute to the reductions in food intake and reward, specifically in females.

Interestingly, estrous cycle phase played an important role in the effects of GLP-1R stimulation in the LH of females. Both behavioral (reward and food intake), and gene expression, changes in this area were dependent on cycle phase, with most potent effects displayed in a high estrogen signaling phase (estrus), and nearly no effect in low estrogen signaling phases (metaestus/diestrus). Hormonal fluctuations within the estrous cycle may therefore drive the differences in the studied behaviors between males and females.

ACUTE PHARMACOLOGICAL BLOCKADE OF GLP-1RS IN THE LH INCREASES FOOD-MOTIVATED BEHAVIOR IN MALES

Intra-LH GLP-1R blockade led to a significant increase in sucrose rewards earned (*Figure 31A*) and active lever presses (*Figure 31B*), only in males. Surprisingly, food intake was unaltered by Ex9 treatment in both sexes (*Figure 31C & D*).

Since GLP-1R stimulation in the LH of females was only effective in the estrus phase, Ex9 treatment was also administered to a separate group of females, only in this specific cycle phase; however, no significant differences of the treatment were found in this experimental group (data not shown). These data suggest that while LH GLP-1R activation seems to be sufficient to reduce food intake and motivated behavior in both sexes, it is only necessary for motivated behavior in males.

That Ex4 exerts a more potent effect on food intake and reward in this area in males is somewhat surprising, since previous results in our lab demonstrated larger effects on these behaviors in females after central Ex4 treatment (Richard et al., 2016). However, more recent data indicate that the LH is not affected by the potentiating effects of estrogen on these behaviors (Vogel et al., 2016); therefore the cycle dependent effects and male/females differences may be due to another cycle-phase dependent hormone.

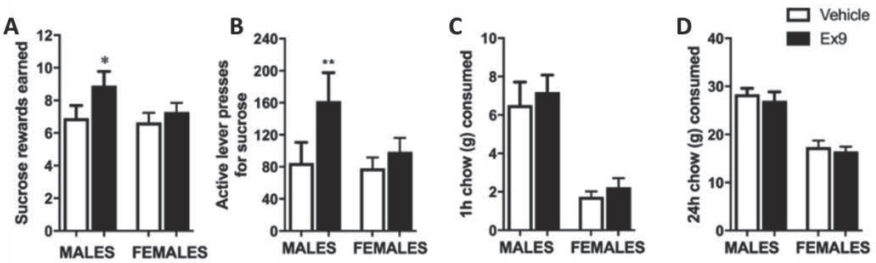

*Figure 31. LH microinjection of GLP-1R antagonist, Ex9 increased the amount of sucrose rewards earned (A), and the number of lever presses for the rewards (B), in a progressive ratio schedule in male rats. None of these parameters were altered in female rats (A & B). Food ingestion, measured at 1 (C) and 24 hours (D), was unaffected in both sexes. Data are expressed as mean ±s.e.m. n=8–10 (male rats) and n=14 (female rats). *p<0.05, **p<0.01, #p=0.07.*

CHRONIC INTRA-LH GLP-1R BLOCKADE INCREASES FOOD INTAKE, BODY WEIGHT AND REWARD PARAMETERS IN MALES, BUT NOT FEMALES

To determine whether chronic GLP-1R blockade alters food intake or food-motivated behavior, GLP-1Rs were knocked down virally in a bilateral manner; this manipulation led to an approximate 50% and 80% reduction in GLP-1R expression in males and females respectively (*Figure 32A & B*).

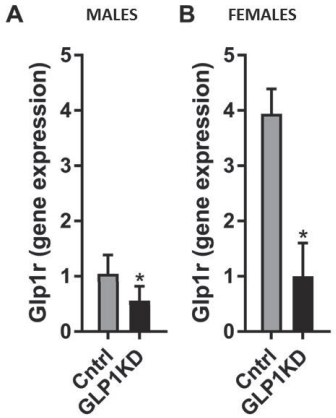

*Figure 32. GLP-1R expression in LH of males (A) and females (B) after site-specific GLP-1R knockdown. Data are expressed as mean ±SEM. n=14–15 (males), n=10 (females). *p<0.05.*

In males, intra-LH GLP-1R knockdown led to a significant increase in food intake (*Figure 33A*) and body weight (*Figure 33B*). In addition, decreased GLP-1R expression in this area increased the fat mass of gonadal and inguinal adipose tissues (*Figure 33C*). Interestingly these results are in contrast to whole body, or CNS specific, GLP-1R knockout studies in mice, where no changes in body weight, food intake or fat mass were reported (Finan et al., 2012; Scrocchi et al., 2000; Sisley et al., 2014). The lack of effects of GLP-1R knockout in these previous studies may be due to species differences or developmental compensation of the knockout in the mice. In addition, since acute pharmacological blockade of intra-LH GLP-1Rs failed to increase these parameters, the effects of acute blockade of these receptors may be compensated for by, for instance, other GLP-1R-expressing sites. Effects of intra-LH knockdown on food intake and body weight were not present in females (*Figure 33D-F*).

Knockdown of GLP-1Rs in the LH also led to a significant increase in sucrose rewards earned (*Figure 33G*), and a trend in active lever presses for reward (*Figure 32H*) in males. Again, no significant effects of intra-LH GLP-1R knockdown on food-motivated behavior were seen in females (*Figure 33I & J*)

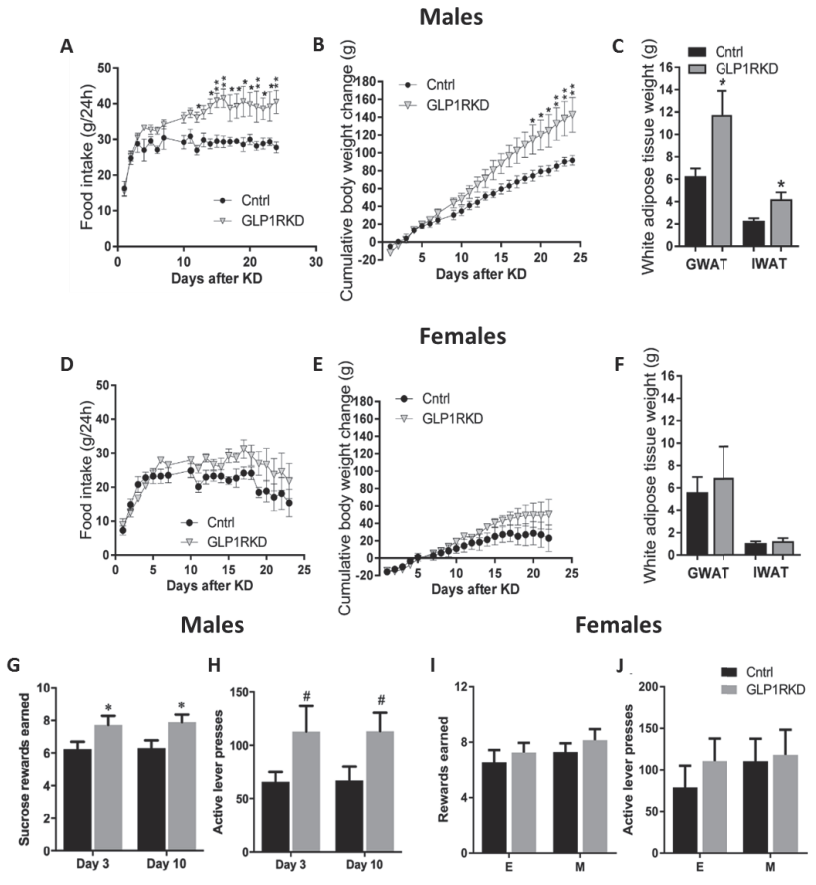

*Figure 33. Chronic blockade of GLP-1 receptors in the LH led to a significant increase in food intake (A), body weight (B) and gonadal (GWAT) and inguinal (IWAT) adipose tissue (C) in males. No significant differences in food intake (D), body weight (E) or fat mass (F) were present after GLP-R knockdown in females. A significant reduction in sucrose rewards earned was present in males (G) after chronic GLP-1R knockdown, while active lever presses for sucrose rewards did not reach significance (H). These parameters were not altered in females (I & J). Data are expressed as mean ±SEM. n=14–15 (males), n=10 (females). *p<0.05, **p<0.01, #p<0.1.*

CONCLUDING REMARKS

Obesity and related conditions pose one of the biggest physical threats on our society today. With sparse treatment options, and the majority of current treatments resulting in suboptimal therapeutic outcomes, the development of more effective pharmaceutical options is required.

The current primary treatment for obesity is the prescription of lifestyle changes, i.e. dietary changes, physical activity and behavioral therapy. It has, however, become evident that enforcing these changes poses many difficulties, leading to low adherence and resulting in no more than 5-10% body weight loss (Wadden et al., 2012).

The most effective weight-loss treatment is currently bariatric surgery, for instance gastric bypass (Roux-en-Y) surgery, a procedure in which the majority of the stomach is bypassed, and food is directed directly to the small intestine. This leads to a reduction in the quantity of food that can be consumed by the individual and the absorption of nutrients and energy, resulting in reduced body weight. Gastric bypass was shown to reduce body weight by approximately 30% in the first year, with patients still maintaining a 20% weight loss 10 years after surgery (Maciejewski et al., 2016). However, gastric bypass surgery also poses a number of short- and long-term risks for the individual, even resulting in death in rare cases. Furthermore, the procedure cannot be applied as a general solution for the ever-growing obese population, as it comes with substantial costs for both the individual and society, making it a suboptimal treatment choice for obese individuals in general.

Apart from weight-loss surgery, there are a number of available pharmaceutical treatments on the market; orlistat (Xenical), lorcaserin (Belviq), phentermine-topiramate (Qsymia) and naltrexone-bupropion (Contrave) are some of the most commonly prescribed. The treatments work through a variety of mechanisms, both central and peripheral. However, these medications only result in an approximate weight-loss of 3-9% (Yanovski and Yanovski, 2014), and increase the risk of a number of side-effects including gastrointestinal issues, nausea, headaches, increased heart rate and/or blood pressure.

In addition to the pharmaceutical treatments mentioned above, the synthetic GLP-1R agonist Liraglutide (Saxenda) was also recently approved for weight-loss treatment. Liraglutide treatment does not exclude the risk of side-effects; it has an increased risk of nausea, diarrhea, constipation, abdominal pain, headaches and increased pulse. While the weight loss effects of this drug are slightly larger than orlistat or lorcaserin, liraglutide treatment only results in a 5-10% reduction in body weight (Mehta et al., 2017). Since GLP-1R agonist treatment is already on the market, and widely used in the obese population, both as a treatment for obesity and type II diabetes, it is of great importance to

investigate the full nature of the peptide on the CNS. In addition, determining the sites responsible for the positive effects of the drug on food intake, body weight and food reward, may enable enhanced outcomes of the drug on these parameters, in addition to circumventing unwanted side-effects.

The included studies identify several new nuclei in the actions of GLP-1R stimulation on food intake and reward, such as the NTS, the PBN and the LH, increasing the understanding of the central mechanisms of the hormone on these behaviors. In addition, site-specific treatment in these areas did not seem to result in nausea, as rodents did not display signs of pica and/or locomotor impairment after GLP-1R agonist administration. The current data therefore identifies several nuclei for future site-specific GLP-1R treatment to reduce the risk of certain side-effects.

Several molecules mediating the anorexigenic and food reward reducing effects of GLP-1 were also identified, for example CGRP, IL-6 and estrogen. Targeted combination of GLP-1R agonist with one or more of these molecules may therefore also facilitate the development of more effective weight-loss treatments. However, more research is necessary to evaluate the safety and effects of combined treatments, in addition to examining the presence of these effects in the human population.

GLP-1-based treatment is prescribed for obesity and type II diabetes in both men and women. However, few preclinical studies have explored the neurobiology and physiology of food-intake and reward regulation in females. Furthermore, drug safety and efficacy have, until recently, not been studied in a sex specific manner, neither in animals or humans (Liu and Mager, 2016). Lack of this knowledge increases the risk of differences in clinical outcome between men and women, i.e. effectiveness and potency of the drug, and differences in incidence and/or severity of side-effects.

Prior to Paper III, little was known about the actions of GLP-1 on reward in females. Together with Paper IV, our data suggest that GLP-1R agonist treatment may have a differential ability to regulate motivated and ingestive behaviors in males and females; in addition, administered synthetic GLP-1 may act in a sex-specific manner on select brain nuclei in the CNS to alter these behaviors. These data therefore highlight the dichotomy between ingestive and food reward studies, where sex differences may be present in one behavior, but absent in the other. In general, our data indicate a higher sensitivity to GLP-1-based substances, centrally in females. However, distinct GLP-1R populations, for instance in the LH, may be less sensitive to actions of the hormone.

While most preclinical studies on GLP-1's effects were previously conducted exclusively in males, clinical studies often include both sexes; regardless these

studies often fail to examine the presence of potential sex differences. In addition, human fMRI studies often fail to test for sex differences as studies are either conducted exclusively in one sex, or the numbers are not powered for detecting differences when both sexes are included. Interestingly, in a recent study examining Ex4's effects in diabetic patients, men and women displayed different sensitivity to the effects of exanatide, where men showed higher sensitivity to the glucose-regulating effects of GLP-1 agonist treatment, while women displayed a higher sensitivity to its weight-loss effects (Anichini et al., 2013). Increased weight loss in females, compared to males, is also in line with our data in Paper III, indicating that sex differences in the effects of GLP-1 agonist treatment may be present across several species, including humans, and not specific to rodents. While further studies in a healthy, obese population are required to determine whether these differences persist in a non-diabetic state, these data suggest that there is a need for sex-specific therapies for treatment with GLP-1 agonists, where differential prescription guidelines, based on indication, may be necessary. In addition, studies investigating potential sex differences in the effects of GLP-1 agonists on food reward in humans are needed, as to my knowledge, this has not yet been investigated.

Though the risk for obesity and/or type II diabetes increases significantly after menopause, to my knowledge, there are no studies investigating potential differences in GLP-1 agonist therapy in pre- versus post-menopausal women. Estrogen, a hormone which is drastically reduced after menopause, was recently shown to increase endogenous GLP-1 production, contributing to estrogen's protective role against type II diabetes (Handgraaf et al., 2018). These data are in line with previous reports demonstrating that pre-menopausal women have higher systemic GLP-1 levels than post-menopausal women, and men (Santos-Marcos et al., 2018). Furthermore, ER expression has been shown to vary based on estrogen level and reproductive state, and central estrogen levels are affected by ovarian estrogen production (Fukuda et al., 2000; Kato et al., 2013; Konkle and McCarthy, 2011; Shughrue et al., 1992). Together with the current data, demonstrating the ability of estrogen to alter the effects of GLP-1 treatment on food reward, GLP-1-based obesity treatment regimens may also have to take menopausal state, and/ or cycle phase, into account.

Beyond diabetes and obesity, GLP-1 agonists are also under evaluation as potential treatments for several other diseases and disorders, including substance abuse. There are prominent gender differences in the prevalence of addiction and substance abuse where men have higher rates of use and dependence of both alcohol and illegal substances (Substance Abuse and Mental Health Services Administration, 2016). However, women have been suggested to be more susceptible to craving, and at higher risk for relapse (Fox et al., 2014;

Hitschfeld et al., 2015; Kennedy et al., 2013; Kippin et al., 2005; Robbins et al., 1999; Rubonis et al., 1994).

Ex4 has previously been shown to reduce alcohol-mediated behaviors and is a promising candidate for alcohol dependence treatment (Egecioglu et al., 2013; Shirazi et al., 2013b; Vallof et al., 2016). In addition, initial experiments from the Jerlhag lab indicate that there may be differences in the effectiveness of GLP-1 analogue treatment between males and females, particularly regarding long-term treatment and relapse after cessation of treatment (manuscript in progress). These data suggest that the sex differences in the effects of GLP-1 based treatment on food reward, demonstrated above, may also be relevant when used to treat other forms of addiction.

Moreover, a series of recently published papers demonstrate that Ex4 attenuates cocaine seeking in rodents; however, none of these studies took sex into account (Hernandez et al., 2018; Hernandez et al., 2019; Schmidt et al., 2016). Women have previously been shown to progress faster to cocaine dependence, and display a greater incidence of relapse (Fox et al., 2014; Robbins et al., 1999). In addition, female rats display a greater preference for cocaine rewards; an effect which has been shown to be estrogen-dependent (Kerstetter et al., 2012; Kerstetter and Kippin, 2011). Thus, future therapies for cocaine addiction may need to take menstrual cycle phase into account.

In addition to addiction, GLP-1 agonists are currently being investigated as a potential treatment for neurodegenerative diseases, such as Alzheimer's and Parkinson's disease. GLP-1 agonist treatment has been shown to exert neuroprotective effects and improve cognitive symptoms, and Ex4 was recently shown to improve motor symptoms in Parkinson's disease (Athauda et al., 2017; Salcedo et al., 2012). Despite that it is well known that sex largely impacts the prevalence, risk factors and outcomes for these diseases (Podcasy and Epperson, 2016), there are currently no studies evaluating potential sex differences in the sensitivity, or outcome of GLP-1-based treatments. Interestingly, sex was previously suggested to affect the treatment response of intranasal insulin in men and women, where the beneficial effect of the drug on cognitive improvement was superior in men (Claxton et al., 2013). Since part of the neuroprotective effect of GLP-1 agonists on neurodegenerative disease is thought to be mediated by improving central insulin signaling (Holscher, 2012), sex differences in the efficacy of GLP-1-based therapies on neurodegeneration may also exist.

Taken together, these data suggest that specific guidelines for GLP-1 agonist treatment may be necessary based on sex and reproductive state. In addition, such guidelines may also be required for future GLP-1 based treatments

targeting other conditions, such as drug addiction or Alzheimer's and Parkinson's disease.

In summary, this thesis identifies three novel sites of action through which GLP-1 mediates its actions on food intake and/or food reward: 1. The NTS, where GLP-1 alters dopaminergic transmission in the VTA by acting on intra-NTS noradrenergic neurons. 2. The PBN, where effects on food intake and body weight are mediated through an increase in CGRP and the cytokine IL-6, and 3. The LH, where GLP-1R stimulation results in a reduction in food intake, body weight and food reward; effects attributed to an increase in the cytokines IL-1 and IL-6. Actions of the hormone in this area are sex specific; necessary and sufficient in males, while solely sufficient in females. In addition, the sex steroid estrogen alters the effects of GLP-1 on reward by acting on ERα-receptors, and females have a higher sensitivity to the food-reward reducing effects of GLP-1 than males. A working model for the combined results is demonstrated in Figure 34.

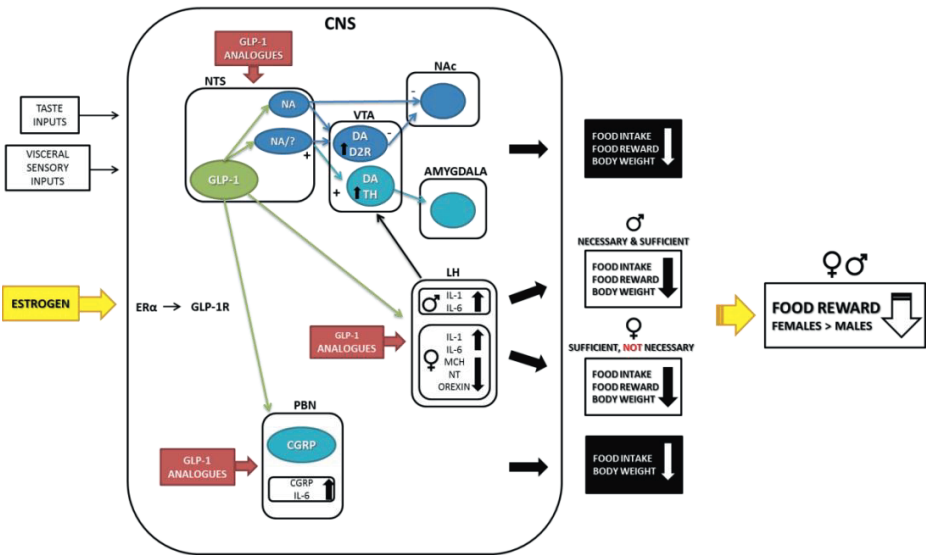

Figure 34. Working model of the effects of GLP-1R stimulation on specific brain nuclei, and sex specific actions, as demonstrated in this thesis. GLP-1 = glucagon-like peptide-1, GLP-1R = GLP-1 receptor, NTS = nucleus of the solitary tract, VTA = ventral tegmental area, NAc = nucleus accumbens, LH = lateral hypothalamus, PBN = parabrachial nucleus, ERa = estrogen receptor alpha, NA = noradrenaline, DA = dopamine, TH = tyrosine hydroxylase, IL-6 = interleukin 6, IL-1 = interleukin 1, MCH = melanocortin-stimulating hormone, NT = neurotensin, CGRP =calcitonin gene-related peptide.

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APPENDIX

I

PLOS | ONE

RESEARCH ARTICLE

Activation of the GLP-1 Receptors in the Nucleus of the Solitary Tract Reduces Food Reward Behavior and Targets the Mesolimbic System

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Abstract

The gut/brain peptide, glucagon like peptide 1 (GLP-1), suppresses food intake by acting on receptors located in key energy balance regulating CNS areas, the hypothalamus or the hindbrain. Moreover, GLP-1 can reduce reward derived from food and motivation to obtain food by acting on its mesolimbic receptors. Together these data suggest a neuroanatomical segregation between homeostatic and reward effects of GLP-1. Here we aim to challenge this view and hypothesize that GLP-1 can regulate food reward behavior by acting directly on the hindbrain, the nucleus of the solitary tract (NTS), GLP-1 receptors (GLP-1R). Using two models of food reward, sucrose progressive ratio operant conditioning and conditioned place preference for food in rats, we show that intra-NTS microinjections of GLP-1 or Exendin-4, a stable analogue of GLP-1, inhibit food reward behavior. When the rats were given a choice between palatable food and chow, intra-NTS Exendin-4 treatment preferentially reduced intake of palatable food but not chow. However, chow intake and body weight were reduced by the NTS GLP-1R activation if chow was offered alone. The NTS GLP-1 activation did not alter general locomotor activity and did not induce nausea, measured by PICA. We further show that GLP-1 fibers are in close apposition to the NTS noradrenergic neurons, which were previously shown to provide a monosynaptic connection between the NTS and the mesolimbic system. Central GLP-1R activation also increased NTS expression of dopamine-β-hydroxylase, a key enzyme in noradrenaline synthesis, indicating a biological link between these two systems. Moreover, NTS GLP-1R activation altered the expression of dopamine-related genes in the ventral tegmental area. These data reveal a food reward-suppressing role of the NTS GLP-1R and indicate that the neurobiological targets underlying food reward control are not limited to the mesolimbic system, instead they are distributed throughout the CNS.

Competing Interests: The authors have declared that no competing interests exist.

Introduction

Feeding behavior is thought to be regulated by two intermingled central nervous system (CNS) pathways: homeostatic and hedonic [1–3]. Hedonic eating is suggested to be one of the main culprits behind the increasing rates of obesity [4]; it is therefore incredibly important to investigate the mechanisms underlying the drive to excessively consume palatable food. Dopamine within the mesolimbic system is considered crucial for the hedonic aspects of feeding behavior [5]. The intake of palatable food increases the activity of dopamine neurons in the ventral tegmental area (VTA) and the release of dopamine in the nucleus accumbens [6,7]. These two mesolimbic nuclei are therefore considered crucial for food reward control and remain the main neuroanatomical sites investigated in studies with a food reward focus. Here, however, we suggest that this may be a limiting perspective and propose that the focus of the food reward regulation studies should be expanded; we suggest that the nucleus of the solitary tract (NTS) in the hindbrain may be an important neural substrate for regulation of food reward.

Glucagon-like peptide-1 (GLP-1) is one of the key signals involved in the CNS regulation of feeding behavior [8–11]. GLP-1 is produced in intestinal L-cells and in the hindbrain, primarily in the NTS [12]. GLP-1-producing neurons innervate the classic homeostatic energy balance-controlling brain regions that include the hypothalamus and the NTS [12–14] and GLP-1 receptors (GLP-1R) have been found in these areas [15]. Hypothalamic and hindbrain GLP-1R have been shown to play an important role in the homeostatic regulation of food intake [16,17]. Stimulation and blockade of these receptors reduces and increases the amount of food eaten respectively [16,17]. However, we have recently demonstrated that in addition to reducing the amount of food eaten central GLP-1R activation also reduces food reward behavior which may represent the hedonic aspect of feeding behavior [18]. This impact of GLP-1R on food reward behavior is thought to be mediated through its actions on the mesolimbic GLP-1R, in areas commonly associated with reward, such as the nucleus accumbens and the VTA [18]. Collectively these findings fit with the view that homeostatic aspects of feeding are regulated by the action of GLP-1 in classically homeostatic areas and reward-driven aspects are controlled by the mesolimbic actions of GLP-1. Here we want to challenge this view by hypothesizing that GLP-1 can impact food reward by acting on its receptors in the hindbrain's NTS. Our hypothesis is strengthened by two recent reports that show that the fat produced hormone leptin and the hypothalamic neuropeptide orexin impact on food reward behavior by acting on their NTS receptors [19,20].

To test the role of NTS GLP-1R stimulation in food-motivated behavior and food reward we utilized two behavioral models typically used to determine the addictive properties of substances, the progressive ratio operant conditioning test and conditioned place preference (CPP) paradigm. The first task is a well-established test of motivated behavior [21], where the higher the motivation to obtain the rewarding substance the harder the rat is willing to work (press a lever) for it. In the conditioned place preference test the more rewarding the animal finds the food, the more time it will spend in the food-associated compartment based on a learned association between the food and the visuospatial cues from the reinforced location. This procedure allows for testing in the absence of food, reducing any potential confounding effects of postingestive feedback during testing. NTS noradrenergic neurons and neurons containing cholecystokinin (CCK) send ascending projections from the NTS to the nucleus accumbens and the VTA providing a possible pathway in which GLP-1 acting within the brainstem can affect the reward system [22]. Therefore in order to determine a potential link between GLP-1 activation with activation of these two neuronal phenotypes, we first evaluated whether central GLP-1R activation changes the gene expression of NTS CCK, and also two enzymes key for noradrenaline production tyrosine hydroxylase (TH) and dopamine-β-hydroxylase

(dBH). We next utilized a transgenic mouse [14] that expresses yellow fluorescent protein (YFP) selectively in the GLP-1-producing neurons to determine whether these neurons innervate TH-positive neurons in the NTS allowing a monosynaptic connection between GLP-1R activation and the mesolimbic system. To further assess the impact of NTS GLP-1R activation on the mesolimbic function we also determined the gene expression of key reward behavior associated genes in the VTA and nucleus accumbens induced by NTS GLP-1R stimulation. Collectively our findings support the notion that the caudal brainstem participates in the control of food reward and food-motivated behavior and identify potential mechanisms via which the NTS GLP-1R-evoked signal may be transmitted to the mesolimbic pathways to reduce food-reward behavior.

Materials and Methods

Animals

Male Sprague-Dawley rats (180–250 g at arrival and 400 g during the drug administration tests, Charles River, Germany) were housed in a 12 h light/dark cycle, in individual cages with free access to chow and water, except during the period of operant testing or peanut butter consumption. Adult female and male mGLU-124 Venus yellow fluorescent protein transgenic mice (YFP-PPG mice; University of Cambridge, United Kingdom [23]) were housed in plastic cages with water and standard chow available ad libitum. All studies were carried out with ethical permissions from the Animal Welfare Committee of the Göteborg University, in accordance with legal requirements of the European Community (Decree 86/609/EEC). All efforts were made to minimize suffering.

Surgery

Rats were implanted with a guide cannula targeting the NTS or the lateral ventricle (26 gauge; Plastics One, Roanoke, VA) under ketamine anesthesia as described previously [24,25]. The following coordinates were chosen for the NTS: ±0.75mm from the midline/on occipital suture/-4.9mm, with injector aimed 6.9 mm ventral to skull; the following coordinates were used for the lateral: ±1.6 from the midline, 0.9 mm posterior to bregma, and 2.0 mm ventral to skull, with injector aimed 4.0 mm ventral to the skull. NTS cannula placement was first verified one week after the surgery by the measurement of the sympathoadrenal-mediated glycemic response by microinjection of 5-thio-D-glucose (24μg/0.3μl) [26]. An elevation of plasma glucose levels by 100% or more was required for subject inclusion in the study. Placement was also assessed histologically *post mortem* by injection of India ink (0.3 μl volume matched drug delivery in the experiments). Only rats whose dye injection site was found within the NTS were included in the data analysis (Fig. 1). The lateral ventricle placement was verified with the angiotensin II drinking test. Angiotensin II was injected at a dose of 20ng in 2μL of aCSF and water intake was measured immediately. Rats that drank at least 5ml of water in 30 min, were considered to have a correct cannula placement.

Drugs

Exendin-4 (Ex4), GLP-1 (7–36) and angiotensin II, were purchased from Tocris (Bristol, UK) dissolved in aCSF (vehicle for all central injections) and stored as aliquots in -20°C.

Palatable food-choice test

Preference for palatable food (peanut butter) or chow was determined by offering the two foods simultaneously on the test day. In order to determine the effect of NTS GLP-1R

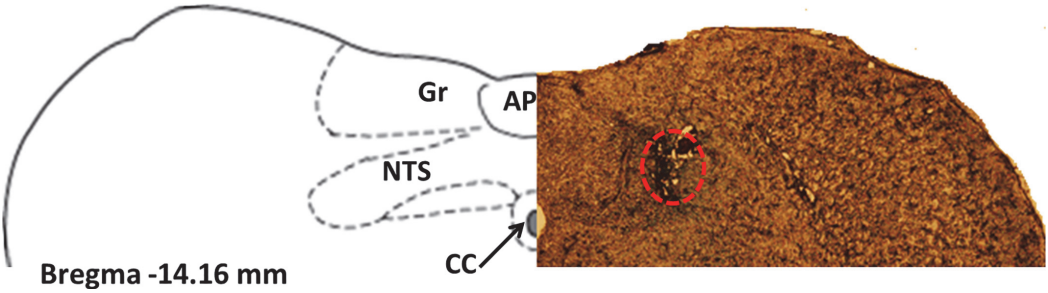

Fig 1. Representative photomicrograph of a coronal section of the rat brain at the level of the NTS illustrating the microinjection site (encircled area) for the behavioral experiments (right panel) and a schematic representation of the NTS from the rat atlas (Paxinos and Watson, 1998) (left panel). Area postrema (AP), central canal (cc), gracile nucleus (Gr).

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activation on the palatable food choice, food presentation was preceded by intra-NTS microinjection of Ex4 (0.05 μg) or aCSF and the amount of chow and peanut butter eaten was measured at 1,3, and 6h. Injections were done in a counterbalanced, Latin square design with at least 48h separating each injection condition. Variations in consumption due to neophobia were reduced by familiarizing all the rats with the peanut butter on at least one occasion prior to the test day. The amount of calories consumed was computed using the following values: 4.1 kcal/g for chow and 6.6 kcal/g for peanut butter.

PICA test

To determine whether Ex4 injections into the NTS are associated with nausea, the PICA response was measured (consumption of non-nutritive substances that mimics emesis in species not capable of the emetic response). Rats were allowed to sample kaolin for at least 3 days before the Ex4 injection to avoid association of kaolin with Ex4 injections. Kaolin intake and chow intake were measured at 1,3,6, and 24h after injection in rats mildly food-restricted overnight (10g of chow available overnight). The dose of Ex4 (0.05 μg) was chosen to reduce chow intake by at least 50%.

Operant conditioning

Food-induced operant conditioning training and testing were conducted in rat conditioning chambers (Med-Associates, Georgia, VT, USA) as described previously [18,27]. Rats were trained to press a lever for a 45mg sucrose reward. Training was conducted in four stages: rats were first trained on the fixed ratio 1 (FR1) schedule (single press on the active lever resulted in the delivery of one sucrose pellet), followed by FR3 and FR5 (3 and 5 presses per pellet respectively), where a minimum of 50 responses per session on the active lever was required for the advancement to the next schedule, culminating with progressive ratio conditioning until stable responding was achieved. Each progressive ratio session lasted for 90 min. Responding was considered stable when the number of pellets earned per session did not differ more than 15% for three consecutive sessions. All operant response testing was performed after the responses stabilized. Rats received drug injections early in the light cycle after partial (10g of chow available) overnight food restriction. This paradigm was chosen to provide a higher motivation to work for sucrose, which was hypothesized to be subsequently attenuated by Ex4 or GLP-1. Injections were done in a counterbalanced, Latin square design with at least 48h separating each

injection condition. Testing was performed during the light cycle. Immediately after the operant boxes test rats were returned to their home cages and offered chow, the amount of chow eaten was measured after 1h and 22h. Body weight was measured at 22h. Locomotor activity was measured during the operant testing with infrared beams installed inside the operant chambers.

Conditioned place preference (CPP)

The CPP test was performed in rats using an apparatus that comprised of two connected chambers with distinct visual and tactile qualities (Med-Associates). Initial preference for one chamber was assessed on the first and second day with 20 min pretests on each day, and the least preferred compartment was determined by taking the average amount of time spent in each compartment across the two pretest days. Subsequently the least preferred compartment was paired with 4g of palatable food (chocolate or peanut butter). The pretest was followed by 16 days of conditioning sessions (2 sessions per day). One day following the last conditioning session rats were injected (intra-NTS) with vehicle (aCSF) or Ex4 (0.05μg) 30min before being placed in the CPP apparatus for 10 min. The behavior of the animals was detected by infrared beams in each chamber and time spent in each compartment was determined. To assure that all palatable food was always consumed during the training sessions the rats had restricted access to chow in their home-cages (to 70% of normal daily chow intake) throughout the CPP experiment.

GLP-1 fiber detection

Mice were anaesthetized with ketamine/xylazine solution and perfused transcardially with heparinized saline followed by fresh fixative solution (paraformaldehyde (PFA, 4%) in 0.1 M phosphate buffer). The brains were collected, and cut into coronal 25 μm sections using a cryostat, sections were then collected into tubes containing tissue storage solution consisting of 50 ml glycerin, 50ml ethylene glycol and 100 ml 0.1 M phosphate buffer (pH 7.5) and stored until use in 4°C. The sections were washed (3 x 15 min) in TNT with Triton-X (0,1%) (Sigma-Aldrich St.Louis, MO, USA). For **tyrosine hydroxylase (TH) visualization**, the sections were incubated for two days in TNB blocking solution (Perkin Elmer, Akron, Ohio, USA) with 1:2000 Goat polyclonal antibody to TH (ab6211, Abcam, Cambridge, UK). The sections were then washed in TNT with Triton-X (0,1%) and incubated in TNB blocking solution with 1:1000 Donkey anti-goat Alexa Fluor 568 (ab36001, Abcam). The cell nuclei were stained with DAPI (1:5000; Life Technologies, Carlsbad, CA, USA). The sections were then washed in TNT (2 x 15 min), submerged in 0.1M PB and mounted on microscope slides (Superfrost Plus, Menzel) together with ProLong Gold Antifade (Life Technologies). The GLP-1 fibers were visualized with a confocal microscope (LSM 700; Carl Zeiss, Oberkochen, Germany).

RNA isolation and mRNA expression

VTA and nucleus accumbens gene expression levels were measured after intra-NTS injection of Ex4 (0.05μg) or vehicle (aCSF). The following genes were examined: *TH*, *Drd1a*, *Drd2*, *Drd3*, *Drd5*, *Slc6a3*, *Gad1*, *Creb1*, *FosB*. They were selected because of their previously reported role in reward behavior regulation or their connection to GLP-1. Ninety minutes after Ex4 or aCSF injection the brains were rapidly removed and the VTA and nucleus accumbens were dissected using a brain matrix, frozen in liquid nitrogen and stored at -80°C. Individual brain samples were homogenized in Qiazol (Qiagen, Hilden, Germany) using a TissueLyzer (Qiagen). Total RNA was extracted using RNeasy Lipid Tissue Mini Kit (Qiagen) with additional DNAse treatment (Qiagen). RNA quality and quantity were assessed by spectrophotometric measurements (Nanodrop 1000, NanoDrop Technologies, USA). For cDNA synthesis iScript cDNA Synthesis

kit (BioRad) was used. Real-time RT PCR was performed using TaqMan probe and primer sets for target genes chosen from an on-line catalogue (Applied Biosystems; reference numbers were as follows: Actb-Rn00667869_m1, TH-Rn00562500_m1, Drd1a-rCG24308, Drd2-rCG57985, Drd3-rCG52650, Drd5-rCG35929, Slc6a3-rCG41956, Gad1-rCG26162, Creb1-rCG22512, Fos-rCG20898). Gene expression values were calculated based on the $^{\Delta\Delta}C_t$ method [28], where the vehicle-injected group was designated as the calibrator. Beta-actin was used as a reference gene. To determine the NTS gene expression of CCK (Rn00563215_m1) and two enzymes key to noradrenaline synthesis, dBH (Rn00565819_m1) and TH, rats were injected into the lateral ventricle with 0.2 μg of Ex4 or vehicle (aCSF) and ninety minutes later an NTS-enriched dorsal brainstem tissue block was dissected. The tissue was rapidly frozen, stored at -80°C, and mRNA extraction, cDNA synthesis and TaqMan PCR were performed as described above. Peptidylprolyl isomerase A (ppia, Rn00690933_m1) was determined to be a more stable gene for the NTS than β-actin, and used as a control gene.

Statistical analysis

All the data are presented as mean ± Standard Error of the Mean (SEM). Statistical significance was analyzed using Student's t test, one- or two-way ANOVA when appropriate (GraphPad Software, Inc., San Diego, CA). P- values lower than 0.05 were considered statistically significant.

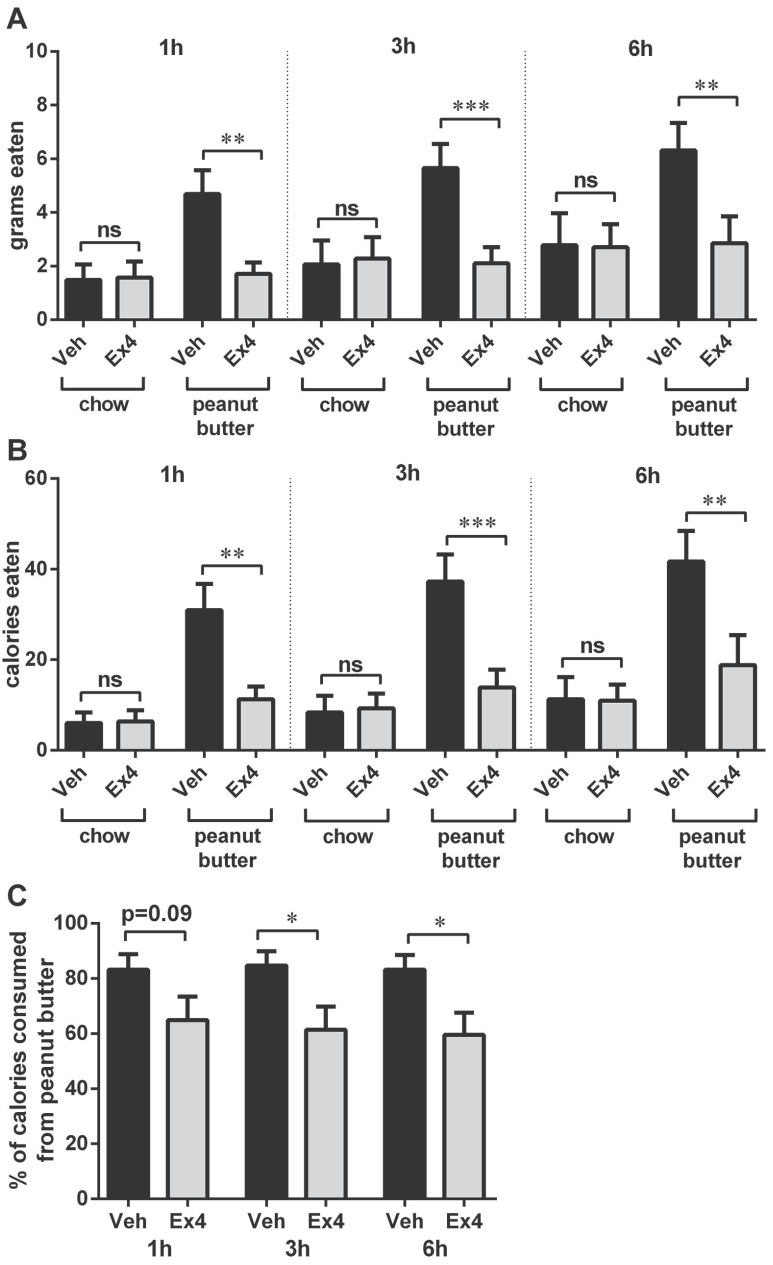
Results

NTS GLP-1R activation preferentially affects intake of palatable food but not chow

Direct NTS GLP-1R stimulation with Ex4 suppressed the intake of palatable food (peanut butter) but not chow *when both were offered simultaneously* (one-way ANOVAs, 1h: $F_{(3,33)} = 8.52$, $p<0.005$; 3h: $F_{(3,33)} = 7.40$, $p<0.01$; 6h: $F_{(3,33)} = 4.42$, $p<0.05$; Fig. 2). The effect of Ex4 was noted at the first, 1h, measurement time point and lasted throughout 6h of measurements. Two-way ANOVAs revealed a significant interaction between the drug treatment and food type at each time point tested (1h: $F_{(1, 11)} = 6.469$, $p<0.05$, 3h: $F_{(1, 11)} = 8.163$, $p<0.05$, 6h: $F_{(1, 11)} = 7.729$, $p<0.05$). Chow intake of vehicle-treated rats comprised 30% of their total intake while 50% of the Ex4 treated animals intake consisted of chow (with respect to amount of grams consumed during the experiment), thus while the intake of chow in vehicle-treated rats was lower than that of peanut butter, it was not negligible. Total amount of calories eaten (chow and peanut butter combined) was as follows: 1h intake vehicle: 37.0±5.9 Ex4:17.7± 4.9 $p<0.0005$; 3h intake vehicle: 45.7±7.3, Ex4:23.2±6.7 $p<0.005$; 6h intake vehicle: 53.0±9.1, Ex4:29.8±9.3 $p<0.05$. Three rats that were determined to have cannula placements not reaching the NTS (injections were localized mostly to the ventral edge of the cerebellum) did not significantly reduce the palatable food or chow intake after Ex4 treatment (vehicle: 9.9±4.2, Ex4: 6.9±2.2 and vehicle: 5.4±2.0, Ex4: 5.3±1.6 for chow and peanut butter respectively; one-way ANOVAs, 6h: $F_{(3,9)} = 1.1$, $p = 0.4$).

Intake of chow when offered alone

However, *when chow was offered as the sole source of food*, *intake of chow was significantly reduced* after intra-NTS Ex4 application (Fig. 3). In this experimental setup the chow intake at 1h (one-way ANOVA: $F_{(2,20)} = 24.12$, $p<0.0001$; Fig. 3A) and 22h (one-way ANOVA: $F_{(2,20)} = 9.56$, $p<0.005$; Fig. 3B) was significantly reduced by both doses of Ex4. The body weight measured at 22h was also significantly reduced after the NTS Ex4 microinjection (one-way ANOVA: $F_{(2,20)} = 4.11$, $p<0.05$; Fig. 3C).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peanut butter
B
calories eaten
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C
% of calories consumed from peanut butter
p=0.09
*
*
100
80
60
40
20
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Fig 2. NTS GLP-1R activation preferentially affects intake of palatable food but not chow. Direct NTS GLP-1R stimulation with Ex4 suppressed the intake of palatable food (peanut butter) but not chow when both were offered simultaneously. The effect of Ex4 had a short latency (noted 1h after injection), and lasted throughout the 6h of measurements. The food intake data are represented as grams eaten (A), as calories consumed, since the two foods differ in their caloric density (B), and as the fraction of total intake (by calories) that was represented by the palatable peanut butter intake(C). Data are expressed as mean ±SEM. n = 12 per each treatment group. * $p<0.05$, ** $p<0.01$, *** $p<0.005$.

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PICA

Intra-NTS administered Ex4 did not induce a PICA response at a dose of Ex4 that potently reduced 1,3,6, and 24h chow intake (nearly a 50% reduction; Fig. 3D). The lack of PICA response, as measured by lack of significant increase in kaolin intake at any time point measured

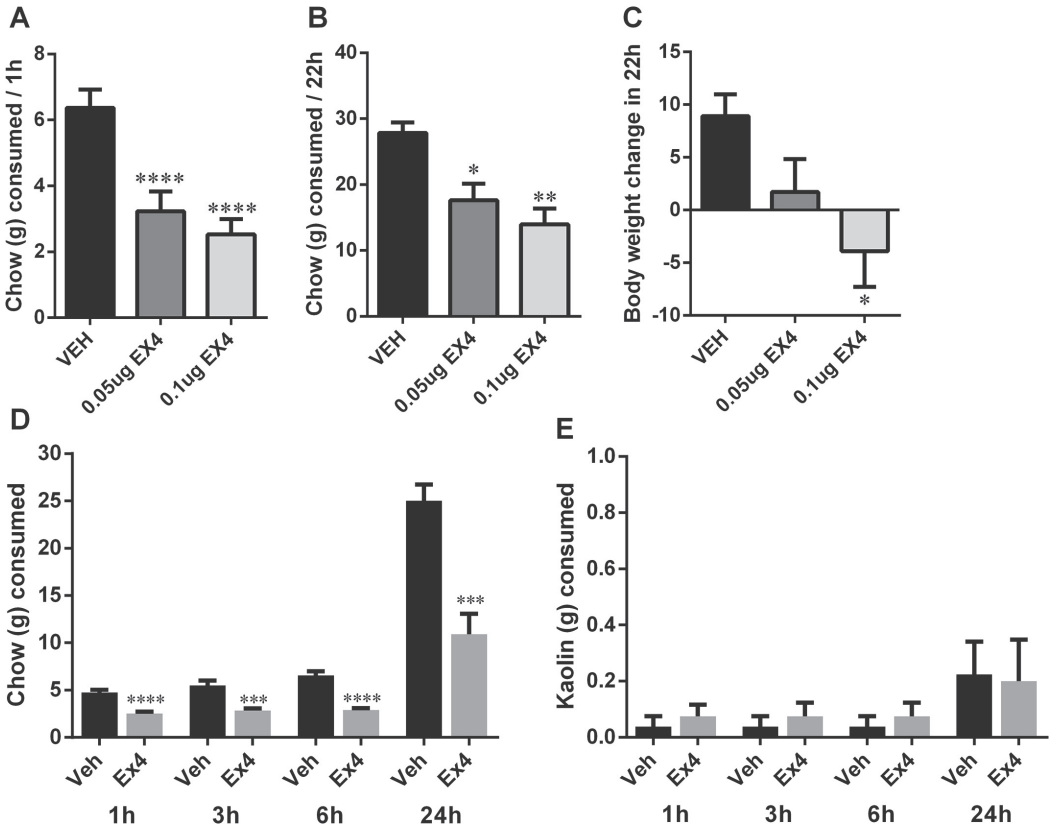

Fig 3. GLP-1R stimulation by Ex4 in the NTS reduces chow intake and body weight. Intra-NTS delivery of Ex4 reduced the consumption of chow over the 22h period of data collection (A-B). Body weight (g) was also reduced 22h after injections (C). In a second group of rats intake of kaolin (PICA response) was measured simultaneously with chow intake. While the chow intake was significantly reduced after intra NTS Ex4 administration (D), intake of kaolin was not altered by Ex4 (E). Data are expressed as mean ±SEM. n = 11 per each treatment group (A-C), n = 8 per each treatment group (D-E). * $p<0.05$, ** $p<0.01$, ***$P < 0.005$, **** $p<0.0005$.

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(Fig. 3E), may indicate that intra-NTS GLP-1R activation, at least at an Ex4 dose of 0.05 μg, is not associated with nausea.

NTS GLP-1R activation decreased food-motivated behavior

Rats responding for a sucrose reward (45mg pellet) under a progressive ratio reinforcement schedule (i.e. a schedule in which the number of presses required to obtain a single sucrose pellet increases progressively) were treated with a selective, long-lasting GLP-1R agonist, Ex4 (0.05 or 0.1 μg/0.3 μl) 10 min prior to placement in the operant boxes. The doses were chosen based on [18]. Intra-NTS administered Ex4 significantly and potently decreased the number of sucrose rewards earned (one-way ANOVA: $F_{(2,20)} = 12.48$, $p<0.0005$; Fig. 4A) and the number of lever presses emitted for sucrose (one-way ANOVA: $F_{(2,20)} = 9.82$, $p<0.005$; Fig. 4B). Post-hoc Tukey tests indicated that both doses of Ex4 were effective at reducing the number of rewards earned and lever presses. These reductions in food-motivated behavior were not accompanied by a general reduction in locomotor activity (Fig. 4C).

Similar results were obtained after intra-NTS application of the endogenous peptide: GLP-1 (2.0 μg/0.3 μl), though the effect of GLP-1 was much less potent compared to that of Ex4. The number of sucrose rewards earned ($p<0.05$; Fig. 5A) and the number of lever presses emitted for sucrose ($p<0.05$; Fig. 5B) were both significantly reduced after GLP-1 treatment. These reductions in food-motivated behavior were not accompanied by a general reduction in locomotor activity (Fig. 5C).

NTS GLP-1R activation decreased food-reward behavior

The food-induced CPP is a complementary test of food reward behavior, in which a rat shows preference for a chamber previously paired with palatable food over a chamber previously paired with no food exposure. The CPP test informs how rewarding the rat finds the palatable food. Importantly, during the CPP test rats do not have access to food enabling dissociation of the intake of palatable food from the reward evaluation process. Rats microinjected with Ex4 (0.05 μg) into the NTS 10 min prior to the CPP test spent significantly less time in the

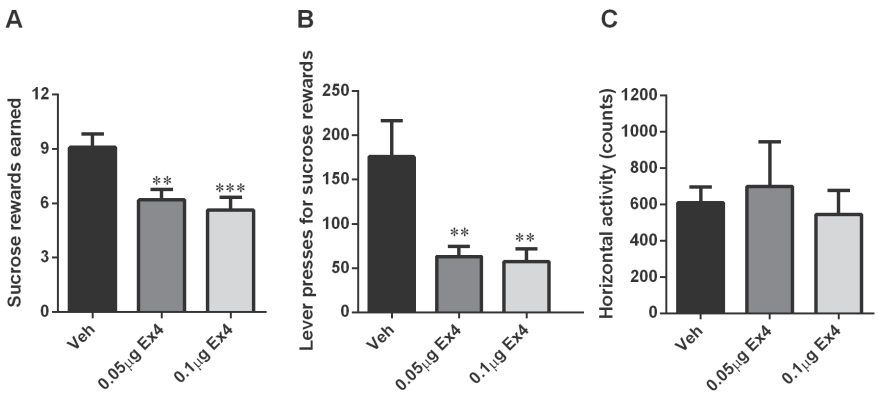

Fig 4. GLP-1R stimulation in the NTS decreased food-motivated behavior. The effect of intra-NTS injection of Ex4 on progressive ratio operant responding for sucrose was tested. Ex4 potently decreased the number of sucrose rewards earned (A) and the number of active lever presses (B) in an operant lever-pressing paradigm. Importantly this suppression in food-motivated behavior was not associated with a reduction in locomotor activity (C). n = 11 per each treatment group. ** $p<0.01$, ***$P < 0.0005$.

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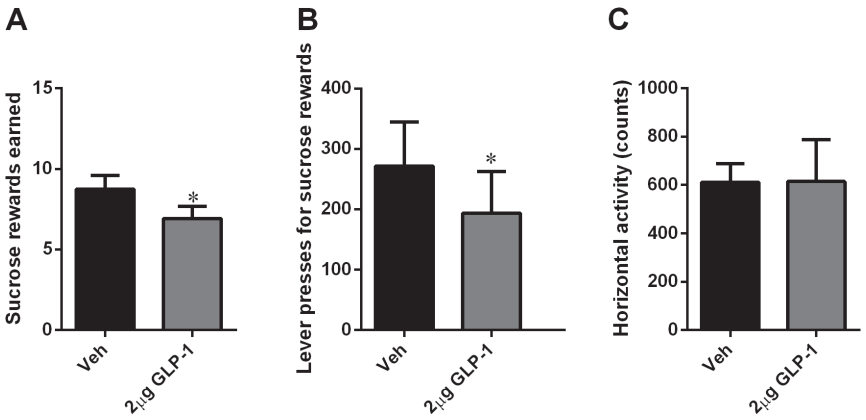

Fig 5. GLP-1 injected into the NTS decreased food-motivated behavior. GLP-1 decreased the number of sucrose rewards earned (A) and the number of active lever presses (B) in an operant lever-pressing paradigm, and this suppression in food-motivated behavior was not associated with a reduction in locomotor activity (C). n = 11 per each treatment group. * $p<0.05$.

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food-paired chamber compared to the vehicle-treated group, and unlike the vehicle treated rats, showed a preference for the non-food paired chamber (Fig. 6).

NTS expression of CCK and two enzymes key in noradrenaline synthesis after central GLP-1R stimulation

Central activation of the GLP-1R with Ex4 increased the expression of dBH (Fig. 7), an enzyme that catalyzes the hydroxylation of dopamine to noradrenaline. The expression of TH, an enzyme that catalyzes an earlier step in the noradrenaline synthesis (the reaction in which L-tyrosine is hydroxylated to obtain L-DOPA) was not altered by Ex4 treatment. The expression of CCK was also unaltered.

GLP-1 fibers closely appose NTS noradrenergic neurons

Guided by previous data showing that neurons expressing the enzyme TH (a marker for catecholamine neurons) in the NTS project to both key mesolimbic nuclei [22,29], the VTA and the nucleus accumbens, we set out to determine whether GLP-1 fibers can be found in close apposition to the NTS TH-positive neurons. TH-immunoreactive neurons that received close appositions from green YFP-positive fibers were found at several levels of the NTS. The TH-positive neurons were most prevalent at the level of the area postrema and just caudally from area postrema (Fig. 8 A-D); at the level of the caudal 4th ventricle the TH-positive neurons were more sparse (Fig. 8 E-F). The distribution of the YFP-labeled cell bodies (Fig. 8 A-D), detected within the caudal lateral NTS and lateral NTS at the level of the area postrema, was consistent with previous literature [11,12]. Few GLP-1-producing cell bodies were also found near the hypoglossal nucleus (Fig. 8C) [14]. Consistent with previous reports [12,29,30], we did not see any colocalization between TH-positive and YFP-labeled cell bodies, which indicated that GLP-1-producing neurons are likely not producing noradrenaline. It should be noted that TH-immunoreactivity within the NTS, particularly in the rostral sections of this nucleus, may also be found in C2 adrenergic neurons in addition to A2 adrenergic neurons. However, since C2 neurons are relatively sparse in the NTS at the level of the area postrema and just caudal to this

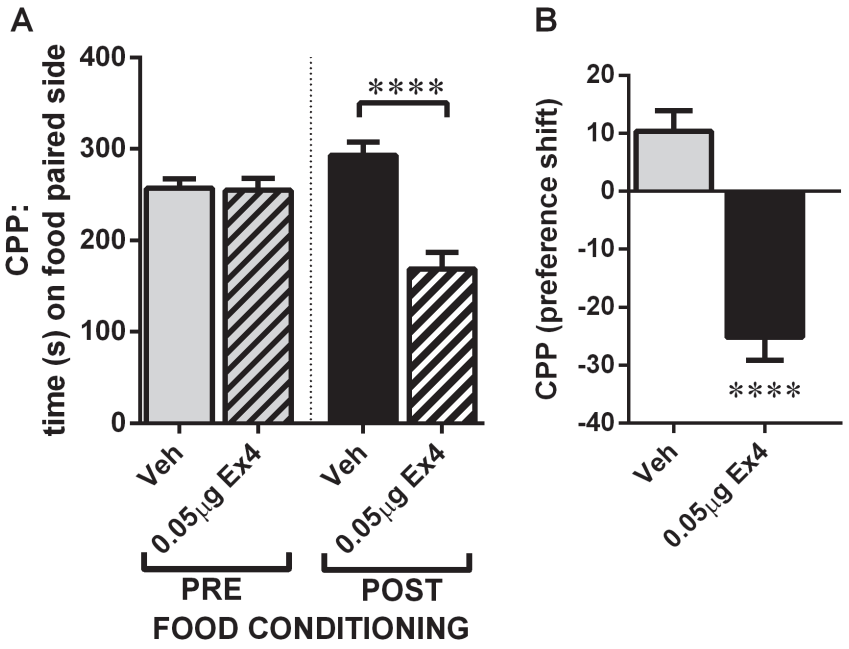

Fig 6. GLP-1R stimulation in the NTS decreased food reward behavior. The effect of intra-NTS injection of Ex4 on the ability of palatable food to condition a place preference was tested. Preference for the chamber paired to palatable food was abolished by Ex4 treatment. The preference [% conditioned place preference (CPP)] was calculated using the following formula: ((test – pre-test)/(total time – pre-test)) × 100. n = 11 (vehicle group) and n = 8 (Ex4 group). ****$p < 0.0005$. Data represent mean ±SEM.

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area, most TH-immunoreactive neurons in this area are noradrenergic, thus allowing the conclusion that GLP-1 fibers closely appose noradrenergic neurons in the NTS.

Mesolimbic gene expression

GLP-1R stimulation in the NTS resulted in a nearly fourfold increase in expression of mRNA encoding TH (Fig. 9A), an enzyme required for the synthesis of dopamine in the VTA and a marker for dopamine neurons in this area. Furthermore, the expression of dopamine 2 receptor (D2R) in the VTA was twofold increased after intra-NTS Ex4 microinjection (Fig. 9A). The expression of other dopamine receptors was not altered (Fig. 9A). Similarly the expression of several other genes previously associated with changes in reward behavior: *FosB*, transcription factor (*Creb1*), as well as the gene encoding glutamate decarboxylase (*Gad1*) remained unchanged after intra-NTS Ex4 treatment in this experimental paradigm (Fig. 9B). Intra-NTS Ex4 treatment did not alter the expression of dopamine receptors, dopamine transporter (DAT), *FosB*, *Gad1* or *Creb1* in the nucleus accumbens (Fig. 9C-D).

Discussion

GLP-1, through its activity on the central receptors, is a key regulator of food intake [8–10,31]. Recently it was also suggested that GLP-1 is essential for control of food reward behavior [18]. Much of the earlier literature reports are focused on neuroanatomical substrates associated

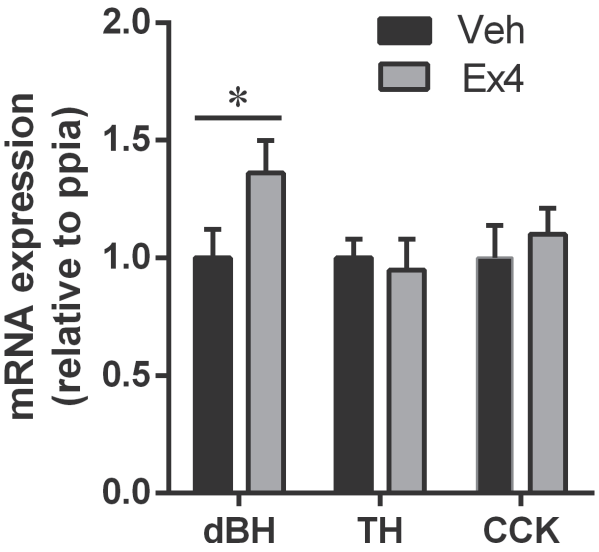

Fig 7. NTS expression of two enzymes key in noradrenaline synthesis after central GLP-1R stimulation. Central activation of GLP-1R with Ex4 increases the expression of dBH but not TH or CCK. Data are expressed as mean ±SEM. n = 9 (pair-fed control group), n = 11 (*ad libitum* fed control group) and n = 10 (Ex4 group). *$p<0.05$. Tyrosine hydroxylase (*TH*) and dopamine-beta-hydroxylase (*dBH*).

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with either homeostatic or hedonic feeding. For GLP-1, the hypothalamus and the hindbrain are considered key sites from which GLP-1 reduces ingestive behavior, while reward effects of GLP-1 are ascribed to the mesolimbic GLP-1R [18,32,33]. Here we report six findings that provide key information about the impact of activation of GLP-1R in the hindbrain, specifically the NTS, on reward behavior: 1) NTS-directed GLP-1R activation resulted in a selective reduction in intake of palatable food. 2) Both GLP-1 and Ex4 microinjected into the NTS suppressed food-motivated behavior for sucrose in a progressive ratio test. 3) NTS GLP-1R activation suppressed food reward behavior in a CPP test. 4) NTS GLP-1-producing neurons are found in close apposition to the NTS noradrenergic neurons, previously shown to send direct projections to the mesolimbic system, specifically the nucleus accumbens and the VTA, providing a potential direct neuroanatomical link between GLP-1 action in the NTS and the mesolimbic system. 5) Central GLP-1R stimulation also increases the NTS expression of dBH, key enzyme in noradrenaline synthesis providing a biological link between GLP-1R activation and NTS noradrenergic neurons. 6) NTS GLP-1R activation altered the expression of dopamine-related genes in the VTA.

Rats given a choice of consuming highly palatable peanut butter or less palatable chow chose to consume nearly four times more calories from the palatable food compared to chow. However, the NTS-directed activation of the GLP-1R attenuated this preference by selectively reducing the amount of palatable food consumed. Thus, it seems that this GLP-1R manipulation selectively affected intake of palatable food. The role for the caudal brainstem, specifically the area postrema and the NTS, in the palatable food-selective ingestive behavior control, was suggested by previous studies utilizing chemical lesions of these regions. Rats with capsaicin-induced neuronal damage to the area postrema and NTS regions overconsumed palatable

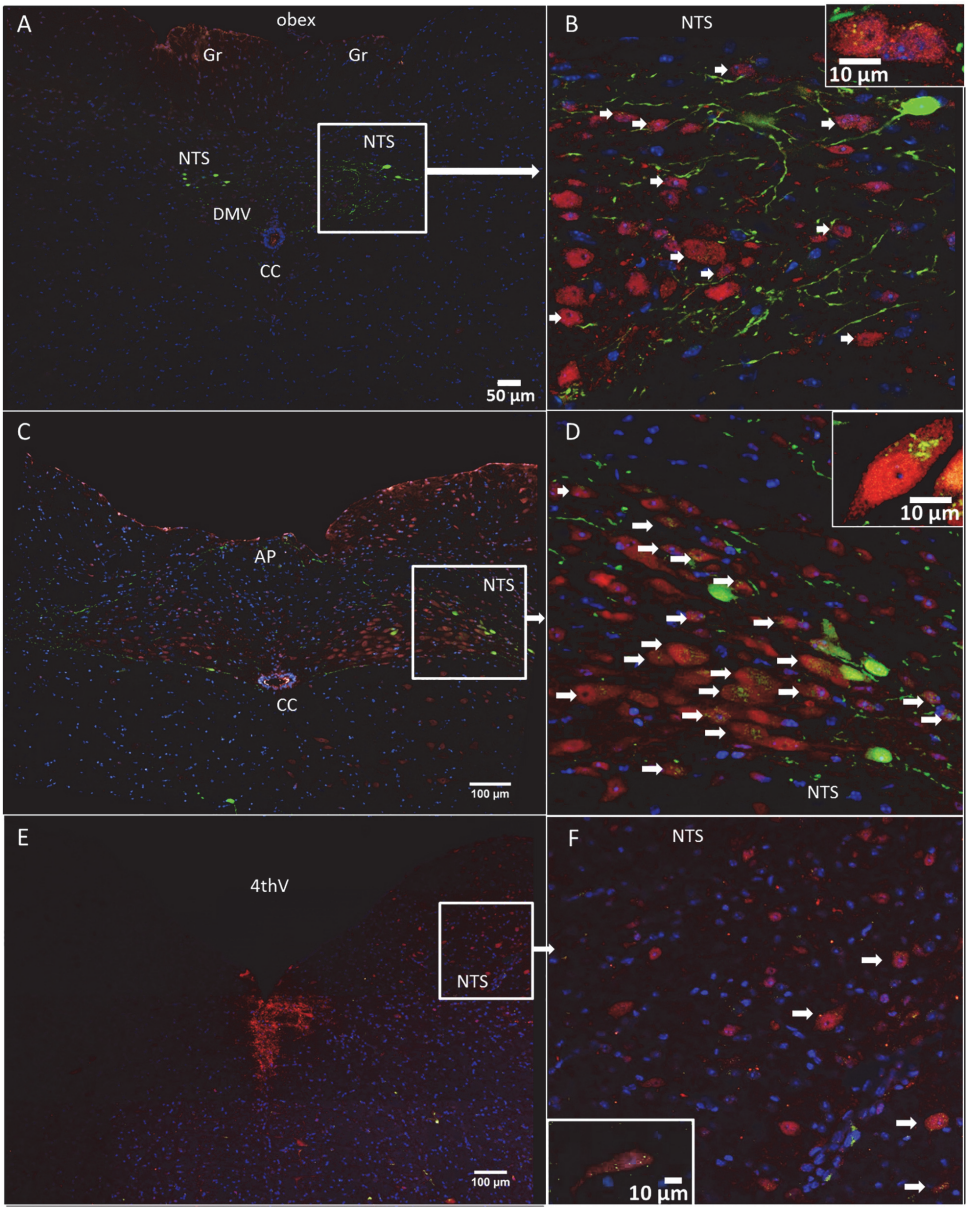

Fig 8. Many YFP-immunoreactive axons (green) closely apposed the TH-positive neurons (red) of the NTS. Fluorescent YFP– preproglucagon neurons (green) and DAPI (nuclear stain, blue) in coronal sections through the NTS of YFP–PPG mice. Micrographs showing the caudal NTS (A-B), the NTS at the level of the area postrema (C-D) and the NTS at the level of the 4th ventricle (E-F). Cell bodies of YFP-immunoreactive preproglucagon neurons (green) were detected at the level of the area postrema and just caudally to the area postrema (A-D). Many green YFP-immunoreactive axons closely appose blue

DAPI-labeled cell bodies in the NTS. White arrows indicate NTS TH-positive neuronal cell bodies closely apposed by the GLP-1 fibers. Insets in panels B,D and F show the interaction at a single neuron level. Area postrema (AP), central canal (cc), dorsal motor nucleus of the vagus (DMV), gracile nucleus (Gr), 4th ventricle (4thV). B,D and F show higher magnification of areas in A,C and D, respectively.

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food, but not chow [34,35]. These rats did not exhibit any other behavioral or physiological changes, underscoring the specificity of this effect. These studies concluded that the capsaicin lesions selectively impair a discrete population of neurons that normally inhibits the exaggerated consumption of preferred food. In light of the current data, an interesting possibility could be that the inputs to the NTS GLP-1-producing neurons, or their downstream neuronal targets within the NTS, may represent one of these discrete populations, inputs to which are disrupted by the hindbrain-injected capsaicin resulting in an exaggerated intake of palatable preferred foods.

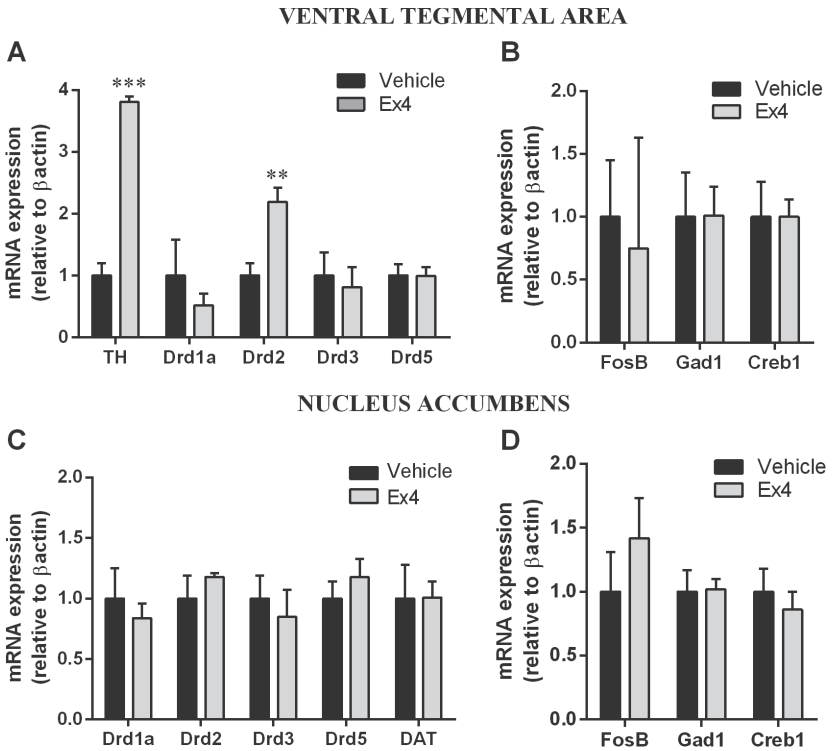

Fig 9. Activation of GLP-1R in the NTS alters gene expression in the mesolimbic reward system. GLP-1R activation by Ex4 in the NTS increased the mRNA expression of the gene that encodes tyrosine hydroxylase (*TH*), and dopamine 2 receptor (*Drd2*) without significantly changing the mRNA expression of other dopamine receptors in the VTA (A). The expression of several other genes previously associated with changes in reward behavior: *FosB*, *Creb1* and *Gad1* remained unchanged after intra-NTS Ex4 treatment (B). Intra-NTS Ex4 treatment did not alter the expression of dopamine receptors, dopamine transporter (DAT), *FosB*, *Gad1* or *Creb1* in the nucleus accumbens (Fig C-D). Data are expressed as mean ±SEM. n = 6 (vehicle group) and n = 5 (Ex4 group). ** $p<0.01$, *** $p<0.005$. Dopamine receptor 1 (*Drd1a*), dopamine receptor 3 (*Drd3*), dopamine receptor 5 (*Drd5*), glutamate decarboxylase 1 (*Gad1*), cAMP responsive element binding protein 1 (*Creb1*), FBJ osteosarcoma viral oncogene B (*FosB*).

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The palatable food selective ingestive changes seen here after intra-NTS GLP-1R activation are also akin to those already demonstrated for central blockade of opioid receptors [for review see: [36,37]]. Similarly to the current results, in a no-choice paradigm (animals are offered only one type of food at a time), opioids change the intake of any kind of food, however when the animals are given a choice opioids selectively alter the intakes of preferred foods [38–40]. There is a neuroanatomical overlap between the opioid and the GLP-1 control of feeding; NTS-selective mu-opioid receptor stimulation and blockade were shown to increase and decrease food intake respectively [41]. The potential functional interaction of the two systems in the hindbrain to control intake of preferred foods is an interesting emerging hypothesis for future investigation. The differential effect of Ex4 on chow intake in a choice vs non-choice food test was already demonstrated in the mesolimbic system, via microinjection of Ex4 into the VTA and the nucleus accumbens shell, where concurrent offering of high-fat and chow resulted in a selective reduction of the high-fat intake [42]. However, when chow was offered alone a potent reduction of chow intake was observed [18]. Since the rats choose to consume more of the palatable food than chow, an alternative explanation to the reduced reward hypothesis could be that Ex4 simply reduces the intake of the principle source of calories. Thus, the food choice experiment alone would not be sufficient to conclude that NTS GLP-1R activation reduces food reward. However, considering that both progressive ratio operant conditioning and CPP for food were reduced by Ex4, both more direct tests of food reward, we conclude that Ex4 does reduce food reward behavior when injected into the NTS. Additionally, in the current study rats consuming chow alone were food restricted to 50% of their regular overnight food intake. Food restriction increases the palatability of any available source of calories, making the consumption of any available food rewarding. Thus, while we cannot eliminate the possibility that mechanisms other than reward suppression are involved in the chow intake suppression after NTS GLP-1R activation, due to the background of food restriction, food reward suppression represents one potential mechanism of reduced chow consumption in the current study.

Two complementary tests of food reward behavior used here, progressive ratio operant conditioning and CPP, clearly demonstrate a role for NTS GLP-1R in food reward suppression. The intra-NTS Ex4 microinjection potently reduced the rats' motivation to work for sucrose pellets. These rats earned 30% less sucrose reward and were willing to put in only a third of the effort (active lever presses) compared to the vehicle-injected control rats. Also in the CPP test, a test that allows complete separation of food consumption and food reward seeking (since no food is offered on the testing day), the intra-NTS Ex4 injected rats showed a suppressed food reward behavior. Unlike control rats, which showed a small but significant increase in the amount of time spent in the environment paired with palatable food during training, the Ex4-treated rats chose to spend significantly more time in the non-food paired environment indicating reduced food-reward seeking behavior. Notably, the changes in reward behavior demonstrated here were not associated with general locomotor inhibition. These results are in line with a recent report that demonstrates that NTS Ex4 can reduce food-reward behavior even 3h after injection [43]. We also report here, for the first time, that the endogenous peptide GLP-1 reduces food reward behavior in the progressive ratio operant task. This is an important finding that strengthens the overall idea of the reward-suppressing role of the central GLP-1 system and attends to the potential criticism posing that the reward-suppressing action of Ex4 is drug-specific. The idea of a differential action of Ex4 and GLP-1 in the brain was suggested by previous studies [44].

We have previously showed that VTA and nucleus accumbens GLP-1R activation with Ex4 reduces food reward behavior [18]. These two mesolimbic sites represent classic neuroanatomical sites accepted for their role in regulation of reward derived from food and drugs of

addiction. Interestingly our current results, combined with the previously reported intra-mesolimbic Ex4 injections, indicate that the NTS GLP-1R were just as sensitive to the reward suppressing action of Ex4 as their VTA and nucleus accumbens counterparts. Thus, even though the caudal brainstem in general, and NTS in particular, is rarely thought of as a reward modulating CNS region, current results clearly call for its addition to the reward-control brain sites.

The mesolimbic circuitry plays a key role in the control of reward behavior. Thus, it is likely that in order for the hindbrain GLP-1R activation to affect reward behavior the NTS GLP-1R-induced activity should be ultimately transmitted to the mesolimbic system. NTS neurons expressing the enzyme TH (a potential label for noradrenergic and adrenergic neurons in the NTS) were previously shown to send direct projections to the nucleus accumbens and the VTA [22,29,45]. In fact NTS A2 neurons are a major source of noradrenaline to the nucleus accumbens and these A2 neurons were already implicated to be crucial in opioid reward and withdrawal behavior [46,47]. Current data indicate that the NTS TH-positive neurons are closely apposed by GLP-1 fibers. Importantly the location of the GLP-1-fiber apposed TH-cell bodies is in line with that previously indicated for nucleus accumbens-projecting TH-positive neurons [22]. These results are in line with one previous study [30]. This neuroanatomical evidence suggests that GLP-1 and activation of GLP-1R in the NTS may reach the key mesolimbic nuclei by changing the activity of the TH-positive neurons. This idea is further supported by our data showing that central GLP-1R activation with Ex4 increases the expression of a key enzyme required for noradrenaline synthesis, dBH. Other possible routes via which GLP-1R activation in the NTS could affect mesolimbic activity may include changing the activity of GLP-1, CCK or neurotensin-positive neurons since all three have been retrogradely labeled from the nucleus accumbens to the NTS [22,42]. The activation of NTS CCK neurons by GLP-1 treatment is not supported by our gene expression data. Also, the activation of GLP-1 neurons by GLP-1R stimulation in the NTS seems unlikely since, at least in a mouse, GLP-1 neurons do not express GLP-1R [48].

Further evidence for the NTS GLP-1R stimulation impacting on activity in the mesolimbic system is provided by current data showing that dopamine-related gene expression in the VTA is altered by NTS GLP-1R stimulation. We show that dopamine 2 receptor (D2R) expression was selectively twofold increased by GLP-1R activation in the NTS, leaving the expression of dopamine 1, 3 and 5 receptor genes unchanged. In the VTA D2R are primarily thought to function as autoreceptors that decrease the activity of the dopaminergic neurons they are expressed by. Interestingly only select populations of dopaminergic neurons may be under this inhibitory control of D2R [49]. Specifically, the majority of the dopaminergic neurons that innervate the nucleus accumbens are inhibited by D2R activation, however nearly none of the dopamine neurons projecting to the amygdala are sensitive to this inhibition [49]. Thus the upregulation of D2R reported here may be consistent with a reduction of the ability of accumbens-projecting dopamine neurons to be activated by reward signals like the palatable food used here. This idea is consistent with the suppression of food-reward behavior. The expression of TH, an enzyme in the VTA associated with an increased production of dopamine, was nearly fourfold increased by the NTS-directed Ex4 treatment. One previous study reported a similar increase in TH protein levels in the VTA after intra-VTA GLP-1R activation [50], thus both GLP-1R populations, in the VTA and in the NTS, despite their neuroanatomical distance, may exert a similar effect on the VTA dopamine production. The projection target/s of the dopaminergic neurons that increase their TH production in response to NTS GLP-1R activation remains to be determined. However, based on previous data it is likely that it is the amygdala. Central GLP-1R activation via lateral ventricle injection of GLP-1R agonist, likely reaching many GLP-1R populations in the brain including those in the VTA and NTS, increases dopamine release in the amygdala [51]. Increased dopamine activity in the amygdala was associated

with reduced food-motivated behavior for sucrose [51]. The case for the amygdala as a likely target may also be strengthened by the fact that amygdala-projecting dopamine neurons would not be inhibited by the simultaneous increase in D2R-mediated inhibition. Alternatively (or in addition to the amygdala release) the increased TH levels in the VTA may contribute to increased somatodendritic release of dopamine in the VTA [52], associated with inhibition of nucleus accumbens and cortex-projecting dopamine neurons.

The reward suppressing GLP-1-driven circuitry outlined here may be engaged by both central and peripheral energy balance controlling signals. Leptin, released from the adipose tissue, has been shown to activate GLP-1 neurons. The anorexic effect of leptin may be partly mediated by GLP-1 [53]. However, just as hindbrain GLP-1R activation may be participating in food reward control, the hindbrain action of leptin may also extend to reward behavior control. Supportively, recent data indicate that activation of leptin receptors in the medial NTS results in a selective reduction in reward derived from food, but not drugs of abuse like morphine [19]. Considering that both hindbrain GLP-1 and leptin receptor activation leads to reward suppression, and the previous studies already show a functional interaction between the two systems. It is possible that the food reward-suppressing effects of leptin are mediated by the central GLP-1 system.

In addition to peripheral signals, NTS neurons may also be engaged by central signals descending from the forebrain in order to regulate food reward behavior. For example orexin, an orexigenic neuropeptide produced in the lateral hypothalamus previously shown to increase food reward behavior [54,55], may be released in the NTS [56,57] to increase the rewarding value of food and food-motivated behavior [20]. Furthermore orexin neurons may innervate the NTS GLP-1 neurons as well as the NTS TH neurons [56]. Thus the two systems, GLP-1 and orexin, may interact within the NTS both by acting on the same downstream neurons (possibly the noradrenergic neurons already shown to link the NTS to the mesolimbic reward system), or alternatively orexin may directly inhibit the GLP-1-producing neurons.

The central GLP-1 system is engaged by signals associated with viscerosensory malaise and formation of consequent conditioned taste aversions, conditions that are associated with reduced food intake. Hindbrain administration of GLP-1 agonists is not associated with conditioned taste aversions [58]. However, medial NTS administration of Ex4 has previously been shown to induce PICA, consumption of non-nutritive substances that may be indicative of viscerosensory malaise [58]. However, the feeding suppression induced by NTS GLP-1R activation does not seem to rely on nausea induction, since at low doses of NTS Ex4, that still potently reduce food intake and reward, our current data and others[43] show that Ex4 does not induce the PICA response. Lack of a sickness response is also supported by the fact that there are no changes in locomotor activity, as sickness responses are typically associated with a reduction in motor activity. Collectively, while it is clear that feeding suppression resulting from NTS GLP-1 stimulation is not always accompanied by nausea, it is entirely possible that with a stronger activation of GLP-1 neurons the NTS GLP-1R activation contributes to reward behavior reduction accompanying visceral malaise. Moreover, NTS GLP-1 neurons are also a key element of the anorexia-inducing neurocircuitry that follows bacterial infection [59]. In fact, NTS noradrenergic neurons are also activated by the bacterial infection mimicking agent-lipopolysaccharide [60]. Thus the NTS GLP-1 to TH circuit suggested by current data may participate in infection-induced anorexia and food-motivated behavior suppression.

Considering the proximity of the NTS to the area postrema with its leaky blood brain barrier it is likely that peripherally injected long-acting GLP-1 analogues utilized clinically (for example Ex4 used here, or liraglutide) may also gain access to the relevant GLP-1R populations to reduce food reward behavior. Thus, the neuronal circuitry identified here may be clinically relevant. Collectively our findings support the idea that the caudal brainstem participates in the

control of food reward and food motivated behavior and identify potential mechanisms via which the NTS GLP-1R-evoked signal may be transmitted to the mesolimbic pathways to reduce food reward behavior.

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Author Contributions

Conceived and designed the experiments: JR RHA KPS. Performed the experiments: JR RHA AG. Analyzed the data: JR RHA KPS. Contributed reagents/materials/analysis tools: FMG FR. Wrote the paper: JR RHA KPS AG FMG FR.

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II

GLP-1 Receptor Stimulation of the Lateral Parabrachial Nucleus Reduces Food Intake: Neuroanatomical, Electrophysiological, and Behavioral Evidence

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The parabrachial nucleus (PBN) is a key nucleus for the regulation of feeding behavior. Inhibitory inputs from the hypothalamus to the PBN play a crucial role in the normal maintenance of feeding behavior, because their loss leads to starvation. Viscerosensory stimuli result in neuronal activation of the PBN. However, the origin and neurochemical identity of the excitatory neuronal input to the PBN remain largely unexplored. Here, we hypothesize that hindbrain glucagon-like peptide 1 (GLP-1) neurons provide excitatory inputs to the PBN, activation of which may lead to a reduction in feeding behavior. Our data, obtained from mice expressing the yellow fluorescent protein in GLP-1-producing neurons, revealed that hindbrain GLP-1-producing neurons project to the lateral PBN (lPBN). Stimulation of lPBN GLP-1 receptors (GLP-1Rs) reduced the intake of chow and palatable food and decreased body weight in rats. It also activated lPBN neurons, reflected by an increase in the number of c-Fos-positive cells in this region. Further support for an excitatory role of GLP-1 in the PBN is provided by electrophysiological studies showing a remarkable increase in firing of lPBN neurons after Exendin-4 application. We show that within the PBN, GLP-1R activation increased gene expression of 2 energy balance regulating peptides, calcitonin gene-related peptide (CGRP) and IL-6. Moreover, nearly 70% of the lPBN GLP-1 fibers innervated lPBN CGRP neurons. Direct intra-lPBN CGRP application resulted in anorexia. Collectively, our molecular, anatomical, electrophysiological, pharmacological, and behavioral data provide evidence for a functional role of the GLP-1R for feeding control in the PBN. **(*Endocrinology* 155: 4356–4367, 2014)**

Glucagon-like peptide 1 (GLP-1), produced in intestinal L-cells and the nucleus of the solitary tract (NTS) in the hindbrain, regulates blood glucose and reduces feeding behavior (1). Much is known about the mechanisms underlying the glucoregulatory function of GLP-1, and this ability of GLP-1 has already been used in the clinic. Genetic and pharmacological data have established that GLP-1 receptor (GLP-1R) activation reduces food intake and, conversely, that a reduction of activity at the GLP-1R increases food intake. Although GLP-1 is a key player in

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Abbreviations: aCSF, artificial cerebrospinal fluid; CCK, cholecystikinin; CGRP, calcitonin gene-related peptide; DAPI, diamidino-2-phenylindole; GABA, γ-aminobutyric acid; GLP-1, glucagon-like peptide 1; GLP-1R, GLP-1 receptor; lPBN, lateral PBN; mPBN, medial PBN; naCSF, normal aCSF; NTS, nucleus of the solitary tract; PB, phosphate buffer; PBN, parabrachial nucleus; PPG, preproglucagon; TNT-buffer, TRIS NaCl Tween 20-buffer; YFP, yellow fluorescent protein.

energy balance control, the mechanisms and neural substrates engaged by GLP-1 to regulate food intake are only beginning to be identified. GLP-1 neurons innervate many brain areas relevant for energy balance control (2, 3). Initially, the literature has emphasized the hypothalamus as the primary target for the feeding inhibition by GLP-1 (4, 5). However, the energy balance control system extends beyond the hypothalamus. Subsequent studies indicate that both local GLP-1 neuronal projections within the NTS and far reaching projections to the mesolimbic ventral tegmental area and the nucleus accumbens are important for the normal regulation of feeding (6–9). In addition, GLP-1R and its mRNA have been identified in the pontine parabrachial nucleus (PBN) (10). Here, we investigate the functional role of GLP-1 in this nucleus for feeding control and the mechanisms involved.

The PBN integrates neural and possibly hormonal signals associated with gustatory properties of food as well as visceral satiety and illness signals. Many neuropeptides central to feeding regulation act on PBN neurons to modulate feeding behavior. Melanocortin and prostaglandin receptor ligands applied directly to the PBN decrease feeding behavior (11, 21), whereas cannabinoid and μ-opioid receptor ligands increase feeding (12, 13). The PBN is a critical nucleus for the creation of taste associations but only in rodents; in primates, and presumably also humans, the PBN mainly functions as a relay and integrator for viscerosensory inputs (14). Recently, interest in the PBN has been rejuvenated by data showing that γ-aminobutyric acid (GABA)ergic and glutamatergic inputs to the PBN are pivotal in the regulation of feeding behavior. When the PBN glutamate/GABA input balance is disturbed, mice stop eating and die of starvation (15–18), a finding that underscores the critical role of the PBN in the regulation of feeding behavior.

The PBN receives direct projections from NTS neurons relaying taste and viscerosensory information in rodents (19), but the neuropeptides that these fibers carry have yet to be elucidated. It is well known that GLP-1 is produced by cell bodies of the NTS and that projections from these cells reach many parts of the brain. Here, using a unique mouse model that expresses a fluorescent protein in GLP-1-producing neurons, we investigate whether these GLP-1 neurons also project to the PBN, thereby providing a source of the endogenous ligand to the PBN GLP-1R. We further evaluated whether the PBN GLP-1R plays a role in feeding behavior control. Because treatments that induce hypophagia drastically increase activity in the lateral PBN (lPBN) neurons, we evaluated whether central GLP-1R stimulation can induce c-Fos protein expression in the PBN and whether GLP-1R activation in the PBN changes the electrical activity of the PBN neurons. Lastly, we identify potential downstream mediators of GLP-1R activation in the PBN, which may include calcitonin gene-related peptide (CGRP) and IL-6. Collectively, our molecular, electrophysiological, pharmacological, and behavioral data provide evidence for a functional role of GLP-1R in the PBN in the control of feeding behavior and identify the neurochemical mechanisms involved.

Materials and Methods

Animals

Adult female and male mGLU-124 Venus yellow fluorescent protein (YFP) transgenic mice (YFP-preproglucagon [PPG] mice; University of Cambridge) (20) were housed in plastic cages with water and standard chow available ad libitum. Male Sprague-Dawley rats (180–250 g at arrival and 450 g during the drug administration tests; Charles River) were housed in a 12-hour light, 12-hour dark cycle, in individual cages with free access to chow and water, except during the period of chocolate and saccharine consumption. All studies were carried out with ethical permissions from the Animal Welfare Committee of the Institute of Experimental Medicine and University of Gothenburg, in accordance with legal requirements of the European Community (decree 86/609/EEC).

Surgery

Rats were implanted with a guide cannula targeting the lPBN or the lateral ventricle (26 gauge; Plastics One) under isofluorane anesthesia as described previously (11, 21). The next coordinates were chosen: lateral ventricle, $\pm 1.6/-0.9/-2.5$ mm (midline/bregma/skull, respectively), with injector aimed 4.5 mm ventral to skull; and lPBN $2.0/-9.5/4.5$ mm, with injector aimed 6.5 mm ventral to skull. PBN cannula placement was verified histologically postmortem by injection of India ink (0.2-μL volume matched drug delivery in the experiments). Only rats whose dye injection site was found within the lPBN were included in the data analysis.

GLP-1 fiber detection

Mice were anesthetized with ketamine/xylazine solution and perfused transcardially with heparinized saline followed by fresh fixative solution (paraformaldehyde 4%) in 0.1M phosphate buffer. The brains were collected, coronal 25-μm sections were cut using a cryostat, then collected in tubes containing tissue storage solution consisting of 50-mL glycerin, 50-mL ethylene glycol, and 100-mL 0.1M phosphate buffer (pH 7.5), and stored until use in 4°C. The sections were washed (3×15 min) in TRIS NaCl Tween 20-buffer (TNT-buffer) with Triton X-100 (0.1%) (Sigma-Aldrich). For CGRP visualization, the sections were incubated for 2 days in TRIS NaCl Boehringer Milk Powder-buffer (TNB-buffer) blocking solution (PerkinElmer) with 1:2000 goat polyclonal antibody to CGRP (ab36001; Abcam). The sections were then washed in TNT with Triton X-100 (0.1%) and incubated in TNB blocking solution with 1:1000 Donkey antigoat Alexa Fluor 568 (ab36001; Abcam). The cell nuclei were stained with diamidino-2-phenylindole (DAPI) (1:5000; Life Technologies). The sections were then washed in TNT (2×15 min),

submerged in 0.1M phosphate buffer (PB), and mounted on microscope slides (Superfrost Plus; Menzel) together with ProLong Gold Antifade (Life Technologies). The GLP-1 fibers were visualized with a confocal microscope (LSM 700; Carl Zeiss). LPBN and medial PBN (mPBN) DAPI-labeled cells and lPBN CGRP-positive cells receiving GLP-1 innervation were quantified from at least 4 25-μm sections per brain. Triple channel confocal images (to cover the entire PBN) were generated with a Plan Fluor $\times$20/0.75 lens and a solid-state laser. A tile scan of 3×3 tiles was obtained from the center of the lPBN and mPBN, respectively. Neurons were considered CGRP-labeled when their staining was clearly above background and their cell nucleus was in the plane of image. Innervation of cells in the PBN by GLP-1 fibers was determined by switching between green- and blue-channel images (for quantification of GLP-1 innervated cells in mPBN and lPBN) and red-, green-, and blue-channel images (for colocalization of GLP-1 fibers with CGRP-labeled neurons).

Food intake and saccharine-drinking measurements after lPBN GLP-1R activation

Consumption of 1) chocolate pellets (n = 11), 2) 0.1% saccharine (n = 12), and 3) chow (n = 11) was measured in 3 groups of rats unilaterally infused (0.2-μL) with a selective and potent GLP-1R agonist Exendin-4 (Ex-4) (0.1- and 1-μg; Tocris) or vehicle (artificial cerebrospinal fluid, aCSF; Tocris) into the lPBN. All injections were performed early in the light cycle. Rats were exposed to both saccharine solution and chocolate pellets on at least 6 occasions before the test to achieve a stable intake and reduce the novelty of the food. Additionally, 24-hour body weight change was measured (n = 11) in the third group of rats. Rats had free access to water at all times and to chow at all times except during the period of chocolate and saccharine intake measurement.

Food intake and body weight measurements after lPBN GLP-1R blockade

Consumption of chow was measured in rats (n = 12) unilaterally infused (0.3 μL) with a selective GLP-1R antagonist Ex-9 (20 μg; Tocris) or vehicle (aCSF; Tocris) into the lPBN. Body weight change was measured overnight, 16 hours after drug injections. Injections were performed 60 minutes before dark onset. Rats had free access to water at all times and to chow at all times except 5 hours before injections (during the light cycle); this was done to ensure equal levels of satiety at the start of the experiment. A similar experimental design was used when testing the effects of intra-lPBN-injected CGRP (3.8 μg; Tocris) or vehicle (aCSF).

Loose-patch clamp electrophysiology

Rats were anesthetized using Isoflurane inhalation. The brain was removed rapidly and immersed in ice-cold sodium-free solution (22). Acute 300-μm-thick coronal slices containing the lPBN were prepared with a VT-1000S vibratome (Leica GmBH) in the sodium-free solution and then equilibrated in normal aCSF (naCSF) (135mM NaCl, 3.5mM KCl, 26mM $NaHCO_3$, 1.2mM $MgSO_4$, 1.25mM NaH_2PO_4, 2.5mM $CaCl_2$, and 10mM glucose, bubbled with O_2/CO_2). Loose-patch clamp measurements to record action currents were carried out as described earlier (23) with slight modifications. Briefly, pipette potential was held at 0 mV, pipette resistance 1–2 MΩ, and resistance of loose-patch seal 7–40 MΩ. The pipette solution contained: 123mM NaCl, 3.5mM KCl, 2.5mM $CaCl_2$, 1.3mM $MgCl_2$, 10mM HEPES, and 10mM glucose (pH 7.3; with NaOH). lPBN was identified under microscopic control, and large ovoid cells of this area (24) were chosen for recordings. Measurements were carried out with an initial control recording (4 min); then in the first experimental group of neurons, Ex-4 (1μM) (25) was added to the naCSF by a single bolus into the recording chamber, and the recording continued for a subsequent 11 minutes. In a second experimental group of neurons, the GLP-1R antagonist Ex-9 (1μM; Tocris) (25) was applied after the initial recording of basal firing. Ex-9 was then present in the naCSF continuously. Ten minutes after starting Ex-9 application, firing was recorded, then Ex-4 was added and the recording continued. Each neuron served as its own control when drug effects were evaluated.

c-Fos expression

Treatment

On the day of experiment, rats were injected with Ex-4 (0.3 μg in 1 μL) or aCSF (1 μL) into the lateral ventricle (n = 3–4 per treatment group). Rats were treated and killed during the light cycle. Rats had ad libitum access to food throughout the study. Ninety minutes after the injections, all of the rats were anesthetized with ketamine-xylazine solution and transcardially perfused with heparinized saline solution, followed by 4% paraformaldehyde in 0.1M PB.

Immunocytochemistry for detection of c-Fos-protein in brain sections

Immunohistochemical detection of c-Fos protein was performed as described previously (26). Briefly, coronal sections (40 μm) were cut on a cryostat through the lPBN, and every third section was collected into PB. Endogenous peroxidases were deactivated, and sections were incubated with a rabbit polyclonal anti-Fos antibody (Ab-5, PC-38 Calbiochem; CN Biosciences UK). Bound antibody was detected with peroxidase-labeled goat antirabbit IgG (Vector Laboratories Ltd) and visualized using a nickel-intensified diaminobenzidine reaction giving a purple-black precipitate within cell nuclei. PBN-containing brainstem sections were viewed under a microscope, and all c-Fos-positive cells were counted in the lPBN by an experimenter blinded to the conditions. Data were expressed as average of total c-Fos counts of all rats; total number per rat was calculated by adding total number of c-Fos-positive cells from the left and right lPBN.

RNA isolation and mRNA expression

PBN gene expression levels were measured after lateral ventricle injection of Ex-4 or vehicle (aCSF) in 2 separate groups of rats. One group was restricted to 10 g ($\sim$50% of average overnight intake) of chow overnight, and the second was allowed to eat ad libitum (n = 6–9 per treatment group). The following genes were examined: *Calca*, *Gabr*, *Gad1*, *Grin2b*, *Il1b*, *Il6*, and *Mc4r*. They were selected because of their reported role in feeding regulation in PBN or their connection to GLP-1. Ninety minutes after Ex-4 or aCSF injection, the brains were rapidly removed, and the PBN was dissected using a brain matrix, frozen in liquid nitrogen, and stored at -80°C. Individual brain samples were homogenized in Qiazol (QIAGEN) using a TissueLyzer (QIAGEN). Total RNA was extracted using RNeasy Lipid Tissue Mini

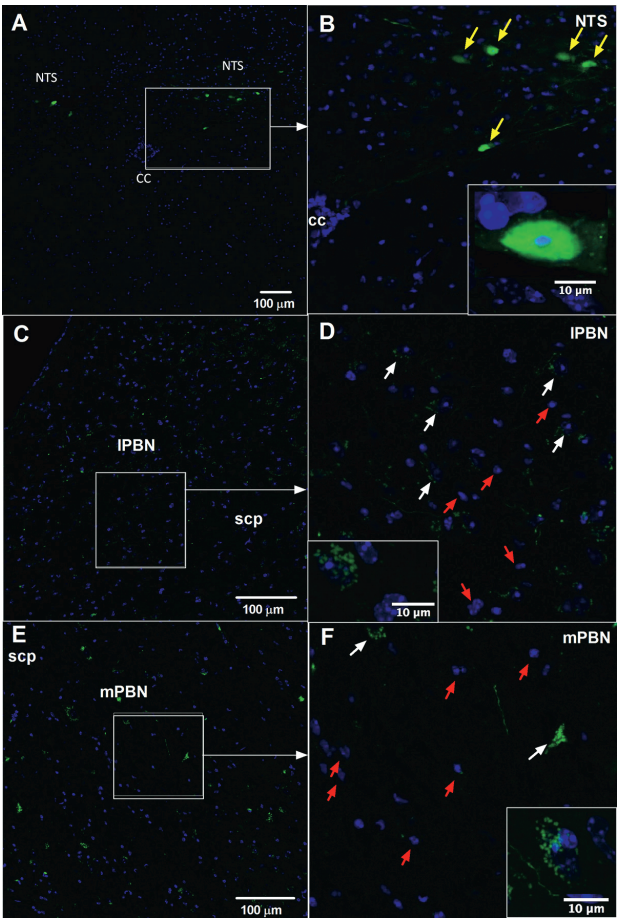

Figure 1. GLP-1 innervation of the PBN. Fluorescent YFP-PPG neurons (green) and DAPI (nuclear stain, blue) in coronal sections through the PBN and the NTS of YFP-PPG mice. Micrographs showing the cell bodies of green YFP-immunoreactive PPG neurons (yellow arrows) in the NTS (A and B). Micrographs showing the lPBN (C and D), and the region of the mPBN just below the superior cerebellar peduncles (scp) (E and F). Many green YFP-immunoreactive axons closely appose blue DAPI-labeled cell bodies in the lPBN. White arrows indicate PBN cell bodies closely apposed by the GLP-1 fibers, whereas red arrows indicate cell bodies in this region that were not apposed by the GLP-1 fibers. Insets in B, D, and F show the interaction at a single cell level. cc, central canal. B, D, and F show higher magnification of areas in A, C, and D, respectively.

kit (QIAGEN) with additional deoxyribonuclease treatment (QIAGEN). RNA quality and quantity were assessed by spectrophotometric measurements (Nanodrop 1000; NanoDrop Technologies). For cDNA synthesis, iScript cDNA Synthesis kit (Bio-Rad) was used. Real-time RT-PCR was performed using TaqMan probe, and primer sets for target genes were chosen from an on-line catalogue (reference numbers were as follows: Actb-Rn00667869_m1, Mc4r-Rn01491866_s1, Calca-Rn01511353_g1, Grin2b-Rn00680474_m1, Gabrd-Rn01517017_g1, Gad1-Rn00690300_m1, IL1b-Rn00580432_m1, and IL6-Rn01410330_m1) (Applied Biosystems). Gene expression values were calculated based on the $\Delta\Delta C_t$ method (27), where the vehicle-injected group was designated as the calibrator (results shown in figure 5 below). β-Actin was used as reference gene.

Statistical analysis

All the data are presented as mean ± SEM. For electrophysiology group, data were expressed as mean ± SEM, and percentage change in the frequency of the firing rate due to the application of the Ex-4 or the Ex-9 was calculated. Each electrophysiological experimental group contained 10 recorded cells from 6–7 animals. Patch clamp recordings were stored and analyzed off-line. Event detection in the recordings was performed using the Clampfit module of PClamp 9.2 software (Molecular Devices Co). For electrophysiology, c-Fos results and feeding data statistical significance was analyzed using Student's *t* test or one- or two-way ANOVA when appropriate (GraphPad Software, Inc). $P < .05$ was considered statistically significant.

Results

GLP-1 fibers in the PBN

Fluorescent YFP-PPG neurons were detected at the caudal region of the NTS of YFP-PPG mice (Figure 1, A and B). Green YFP-immunoreactive axons were found to closely appose blue DAPI-labeled cell bodies in the lPBN (Figure 1, C and D). Over half (55 ± 1.2%) of the lPBN cell bodies were found to receive fibers from the hindbrain GLP-1 neurons. The medial region of the PBN was also found to receive YFP-immunoreactive fibers, however to a lesser extent than the lPBN region (31 ± 2.3% of the DAPI-positive mPBN cells were innervated by GLP-1 fibers) (Figure 1, E and F).

Food intake, saccharine drinking, and body weight after intra-lPBN GLP-1R stimulation

The goal of the in vivo experiments was to determine whether GLP-1R activation in the PBN can contribute to food intake reduction across a variety of caloric, palatable and less palatable, as well as noncaloric sweet liquid foods. PBN GLP-1R stimulation via microinjection of a selective GLP-1R agonist Ex-4 significantly reduced chocolate pellet consumption over the 2-hour period of measurement

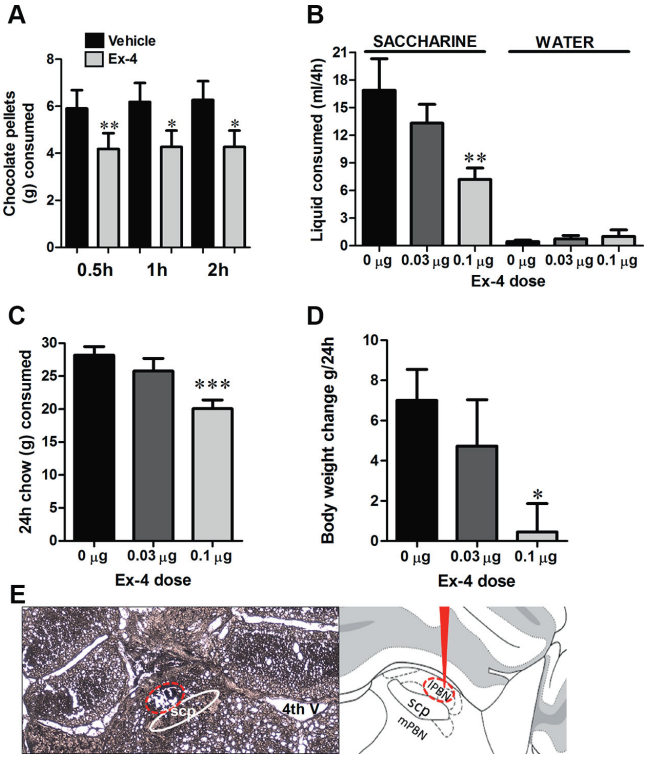

Figure 2. GLP-1R stimulation by Ex-4 in the lPBN reduces food intake and body weight. Intra-lPBN delivery of Ex-4 reduced the consumption of chocolate pellets over the 2-hour period of data collection (A), the amount of saccharine drank (but not water consumption) over 4 hours of data collection (B), the 24-hour chow intake (C), and 24-hour body weight change (D). Data are expressed as mean ± SEM. *, $P < .05$; **, $P < .01$; ***, $P < .005$. E, Representative photomicrograph of a coronal section of rat brain at the level of the lPBN illustrating the microinjection site (encircled area) for the behavioral experiments (left panel) and a schematic representation of the PBN (right panel). scp, superior cerebellar peduncles; 4th V, 4th ventricle.

(Figure 2A). Intra-PBN Ex-4 microinjection also reduced the amount of saccharine drunk by a separate group of rats (one-way ANOVA, $F_{(2,35)} = 5.96$; $P < .008$) (Figure 2B) without affecting water drinking (offered in parallel with saccharine). Moreover, the intake of normal chow (one-way ANOVA, $F_{(2,38)} = 9.19$; $P < .001$) (Figure 2C) and body weight (one-way ANOVA, $F_{(2,35)} = 10.5$; $P < .001$) (Figure 2D) were also reduced when measured over a 24-hour period.

Food intake and body weight after intra-lPBN GLP-1R blockade

Blockade of lPBN GLP-1Rs resulted in a significant increase in chow intake at 2 and 3 hours after Ex-9 injections and a significant increase in body weight measured overnight 16 hours after Ex-9 injection (Figure 3, A–C).

PBN GLP-1R stimulation results in increased firing rate of lPBN neurons

To test the hypothesis that Ex-4 influences function of large ovoid neurons in the lPBN, we examined the electrophysiological response of these neurons to Ex-4. In the first experimental group, Ex-4 (1μM) was applied and increased the firing rate significantly (330 ± 60% of the control) (Figure 4, A and B). The basal firing rate (without any drugs) was 1.02 ± 0.44 Hz (Figure 4A). In a second experimental group, Ex-4 was administered in the presence of the GLP-1 antagonist, Ex-9 (1μM), and the firing rate remained unaltered (135 ± 52% from the firing rate obtained with Ex-9) (Figure 4, C and D). This value was, however, significantly different from that achieved with Ex-4 alone (Figure 4E). Application of Ex-9 alone did not affect the firing rate of the recorded neuron (115 ± 34% of the basal firing rate).

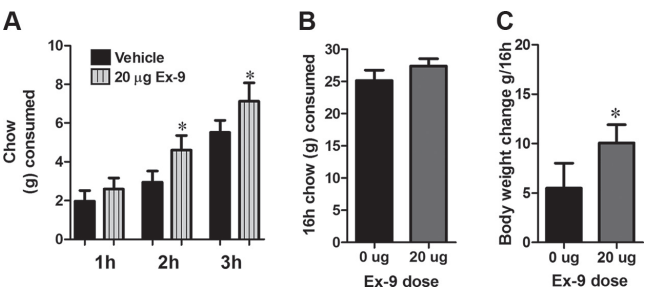

Figure 3. GLP-1R blockade in the lPBN increases food intake and body weight. Intra-lPBN delivery of Ex-9 increased chow intake at 2 and 3 hours after injection (A). Overnight chow intake measured at 16 hours after Ex-9 injection was not altered (B). Ex-9 increased 16-hour body weight gain. C, Data are expressed as mean ± SEM. *, $P < .05$.

c-Fos protein expression

To confirm the electrophysiology results from rat PBN slices in vivo, we determined whether a central injection of Ex-4 can activate PBN neurons. Central GLP-1R stimulation via lateral ventricle injection of Ex-4 increased the

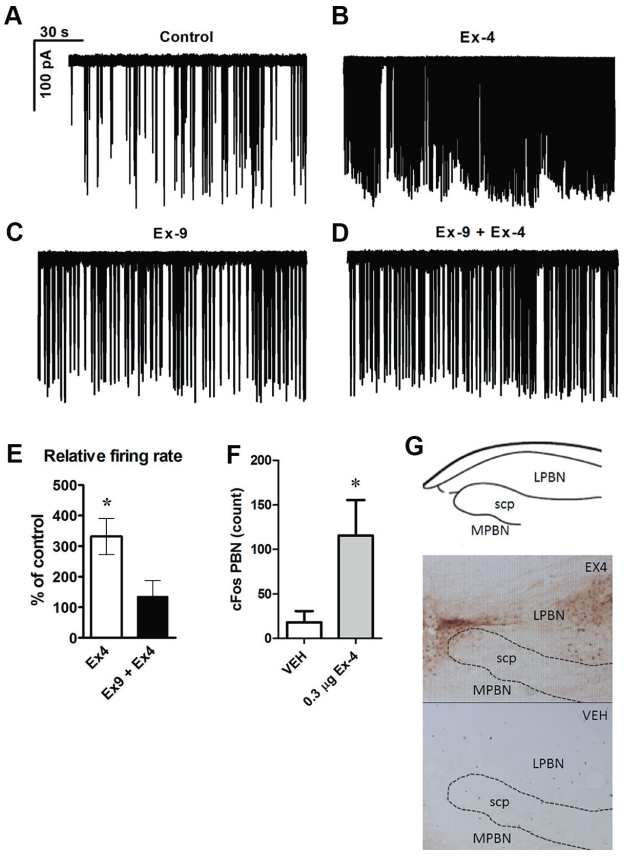

Figure 4. Loose-patch clamp recordings of action currents in the neurons of the external lPBN. Application of GLP-1R agonist Ex-4 (1 μM) in the extracellular solution increased the firing rate (A and B). Extracellular administration of the GLP-1R antagonist Exendin-9(9-39) (Ex-9) (1 μM), blocked this effect of Ex-4 (C and D). Histogram shows the relative percentages of firing rate after application of Ex-4 with and without Ex-9 (E). Central GLP-1R stimulation by lateral ventricle injection of Ex-4 increased c-Fos activation in the PBN. Quantified immunoreactivity of Fos-positive neurons in the PBN after Ex-4 treatment in ad libitum-fed rats (F) and representative images of the c-Fos study (G). Data are expressed as mean ± SEM. c-Fos data were expressed as average of total c-Fos count of all rats; total number per rat was calculated by adding total number of c-Fos-positive cells from the left and right lPBN. Each electrophysiological experimental group contained 10 recorded cells from 6–7 animals. *, $P < .05$. scp, superior cerebellar peduncles; VEH, vehicle.

number of detected c-Fos-positive cells in the lPBN (Figure 4, F and G).

Gene expression

Central activation of GLP-1Rs resulted in a 62% increase in expression of mRNA encoding CGRP (*Calca*) (Figure 5A), an anorexic peptide that is expressed in the intra PBN- and amygdala-projecting PBN neurons, in ad libitum-fed rats. The expression of the gene encoding melanocortin receptor 4, the stimulation of which in lPBN was previously shown to result in anorexia, was not altered. Similarly, the expression of genes encoding receptors for N-methyl D-aspartate receptor subtype 2B (*Grin2b*) and GABA-A receptor δ (*Gabrd*), as well as the gene encoding glutamate decarboxylase (*Gad1*), remained unchanged after Ex-4 treatment in this experimental paradigm. We next determined whether Ex-4 treatment increased the expression of *IL1*β and *IL6*, 2 molecules that mediate a part of the anorexic effects of Ex-4 in the hypothalamus and the hindbrain (28), and showed that Ex-4 increased *IL6*, but not *IL1*β, gene expression in the PBN in both ad libitum-fed and food-deprived rats (Figure 5, C and D). The next average ΔCt values (±SEM) relative to β-actin were detected for ad libitum-fed rats: *Calca* (3.0 ± 0.3, 2.3 ± 0.1, $P < .01$), *Gabrd* (5.6 ± 0.2, 5.6 ± 0.2), *Gad1* (5.2 ± 0.2, 5.3 ± 0.1), *Grin2b* (6.6 ± 0.2, 6.4 ± 0.1), *Mc4r* (9.0 ± 0.3, 8.7 ± 0.2), *IL1* β (9.2 ± 0.2, 8.9 ± 0.7), and *IL6* (11.9 ± 0.2, 9.4 ± 0.7) ($P < .005$); values are given for vehicle and Ex-4, respectively. The next average ΔCt values relative to β-actin were detected for overnight food-restricted rats: *Calca* (2.6 ± 0.2, 2.7 ± 0.1), *Gabrd* (5.9 ± 0.3, 5.2 ± 0.3), *Gad1* (5.6 ± 0.3, 5.3 ± 0.2), *Grin2b* (6.3 ± 0.2, 6.4 ± 0.1), *Mc4r* (8.9 ± 0.2, 8.9 ± 0.1), *IL1*β (8.5 ± 0.1, 8.1 ± 0.4), and *IL6* (11.3 ± 0.2, 9.1 ± 0.5) ($P < .005$); values are given for vehicle and Ex-4, respectively.

Innervation of lPBN CGRP-positive cells and effect of intra-lPBN CGRP injections on food intake and body weight

Guided by results indicating an elevation in CGRP in the PBN, we set out to determine whether the GLP-1 fibers provide direct innervation to the lPBN CGRP neurons. Our results indicate that most lPBN-projecting GLP-1 fibers innervate CGRP-positive cells, and nearly half of the CGRP-expressing cells in the lPBN receive GLP-1 innervation (Figure 6, A–D). Furthermore, we

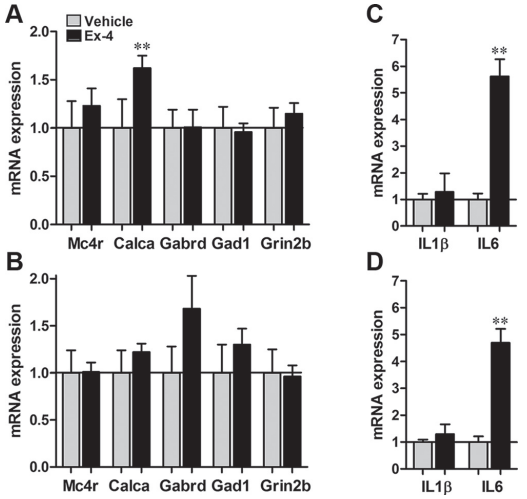

Figure 5. Gene expression after central GLP-1R stimulation. In ad libitum-fed rats, GLP-1R activation by Ex-4 increased the mRNA expression of the gene that encodes CGRP (*Calca*), without significantly changing the mRNA expression of other genes previously shown to be associated with changes in food intake in the PBN (A). In overnight-fasted rats, Ex-4 did not significantly change the mRNA expression of any of the genes measured (B). Ex-4 increased the expression of *IL6* (but not *IL1β*), central mediators of GLP-1R-induced anorexia in both ad libitum-fed (C) and fasted (D) rats. Data are expressed as mean ± SEM. **, $P < .01$.

determined whether elevated CGRP is sufficient to alter food intake when applied directly and selectively to the lPBN. Intra-lPBN CGRP injections resulted in a significant short-term (1–2 h) food intake reduction (Figure 7A). Food intake and body weight at 16 hours after CGRP injections were not altered (Figure 7, B and C).

Discussion

Viscerosensory stimuli result in neuronal activation of the PBN, but the origin and neurochemical identity of the excitatory neuronal input to the PBN remain largely unexplored. Here, we provide data implicating NTS-originating GLP-1-producing neurons as one source of excitatory projections to the PBN. Moreover, we demonstrate a functional role for parabrachial GLP-1R activation in food intake control. Several lines of evidence support this conclusion. Direct activation of lPBN GLP-1R inhibited food intake and body weight gain, and conversely lPBN GLP-1R blockade increased food intake and body weight gain in rats. These data indicate that lPBN GLP-1R are necessary and sufficient for food intake control. Stimulation of PBN GLP-1R potently activated PBN neurons, the activation of which has previously been linked to anorexia. The activation of lPBN neurons via GLP-1R is underscored both by an activational effect shown via electrophysiology in rat brain slices, and as increased activity of PBN neurons, reflected by a significant increase in the number of c-Fos-positive cells in the lPBN after central Ex-4 injection. Moreover, our neuroanatomical data implicate solitary tract GLP-1 neurons as the source of endogenous agonist for the GLP-1R in the lPBN. Using a unique and well-validated Venus PPG reporter mouse (3, 20, 29, 30), we show GLP-1-containing fibers in the lPBN that are very likely to originate from the NTS, the only major source of GLP-1-producing neurons in the brain. We also identify the downstream neurochemical mechanism of the anorexic effect of GLP-1 in the lPBN. We show that in lPBN, GLP-1R activation increased the expression of CGRP, and most NTS-originating GLP-1 fibers innervated lPBN CGRP-producing neurons. Moreover, increased CGRP signaling in the lPBN induced anorexia and body weight reduction.

Stimulation of GLP-1R in the PBN increased the activity of neurons in this area, as implied by our electrophysiology and c-Fos data. These data fit remarkably well with previous studies showing that activation of neurons in the PBN, by removal of the hypothalamic inhibitory GABAergic projections to the PBN or by optogenetic activation of lPBN CGRP-expressing neurons, resulted in hypophagia in mice (17, 18). Here, we propose that activation of lPBN neurons by GLP-1 may be one of the excitatory mechanisms that are uncovered when the hypothalamic brake on the PBN neurons is removed. It is likely, however, that GLP-1 transmission is not the only excitatory input to the PBN. Glutamatergic input may also be involved, because knockdown of N-methyl-D-aspartate glutamate receptors in the PBN can prevent the hypophagia resulting from GABAergic signal removal (16). Moreover, GLP-1-producing neurons may also produce a fast neurotransmitter, which could be glutamate. Even though Ex-4, a potent and selective agonist for GLP-1R, was used in the current study our results are likely relevant for the GLP-1 peptide. In fact, one previous report already indicated that central injections of the native peptide, GLP-1, at the start of the dark cycle in rats also induces c-Fos in the PBN (31).

Until recently, detection of the distribution of axonal fibers of the GLP-1 neurons was hindered by the need to use antibodies with neuronal tracers. This process was facilitated by the creation of a transgenic mouse that expresses a fluorescent signal, YFP, in PPG-expressing cells; this mouse model has been used to identify GLP-1-producing neurons in the brain (3, 20, 29, 30). The transgenic YFP-PPG mice offer an advantage over previous methods in the form of the strong YFP expression that allows for clear visualization of the GLP-1-producing neuron axon

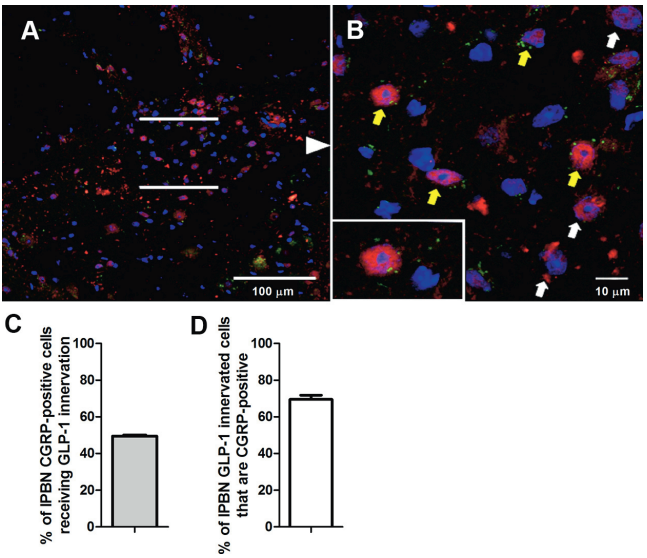

Figure 6. GLP-1 innervation of CGRP neurons in the lPBN. Many YFP-immunoreactive axons (green) closely apposed the CGRP neurons (red) of the lPBN. Yellow arrows indicate CGRP-labeled lPBN cell bodies closely apposed by the GLP-1 fibers, whereas white arrows indicate CGRP-labeled cell bodies in this region that were not apposed by the GLP-1 fibers. B, Higher magnification of the lPBN region presented in A. Inset in B shows the interaction at a single cell level. Blue color represents DAPI the nuclear stain. Nearly half of the CGRP-positive cells in the lPBN receive GLP-1 innervation (C), and most cells in the lPBN that were innervated by GLP-1 fibers were CGRP-positive (D).

fibers and terminals. We detected dense YFP-positive axons at several levels of the PBN. GLP-1 innervation was detected throughout the rostro-caudal extent of the PBN, with denser innervation detected in the rostral region. YFP-positive fibers were also found in the dorso-lateral PBN, the external-lateral PBN, and the mPBN. Thus, we show neuroanatomical grounds for NTS GLP-1 neuron communication to all levels of the PBN. This potentially allows GLP-1 to influence a wide range of physiological responses controlled by different nuclei of the PBN. One potential downside of using the YFP mice in the current study is that there may be a species difference in the projection targets of the GLP-1-producing neurons. For example, leptin control of GLP-1 neurons has been shown to differ between mice and rats (32). In mice, leptin receptors are located directly on the GLP-1-producing neurons in the NTS, whereas in rats, leptin may only be influencing GLP-1 neuron activity indirectly (32). Nonetheless, some literature already exists to support a direct, monosynaptic, connection between the caudal NTS and the PBN in a rat. Indeed, it seems that PBN, especially the lateral subdivisions, receives very dense innervation from the NTS also in the rat (2). Innervation from the NTS was shown to overlap with GLP-1-positive terminals, and a retrograde tracer injected into the lPBN was indicated to colocalize with NTS GLP-1-producing neurons in a rat (2, 33). Combined with other data showing expression of GLP-1R in the rat PBN (10) and the strong behavioral effect of GLP-1R activation in this species, direct projection to the PBN from the NTS seems rather likely in the rat.

GLP-1 activation in the lPBN suppressed chow intake, intake of palatable chocolate pellets, and also intake of noncaloric saccharine solution. This indicates that GLP-1R signaling in the lPBN can reduce food intake across the palatability and caloric density spectrum. Importantly PBN Ex-4 injections did not reduce water intake. Collectively, these data indicate that PBN GLP-1 signaling may interact with the caloric density, taste, and hedonic properties of food. The PBN is a crucial relay for the hedonic value of food; lesion of the PBN blunts nucleus accumbens dopamine elevation in response to palatable food (34). Moreover, one recent study indicates that lPBN-directed Ex-4 injections reduce high-fat food intake and suppress the motivation to work for a high-fat reward in rats (33).

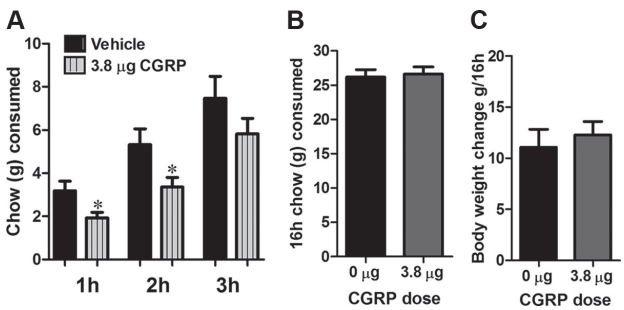

Figure 7. CGRP reduces food intake via a direct action in lPBN. Intra-lPBN delivery of CGRP reduced chow consumption up to 2 hours after injections (A). This effect was short lasting, because chow intake (B) and body weight (C) measured 16 hours after injections were not altered. Data are expressed as mean ± SEM. *, $P < .05$.

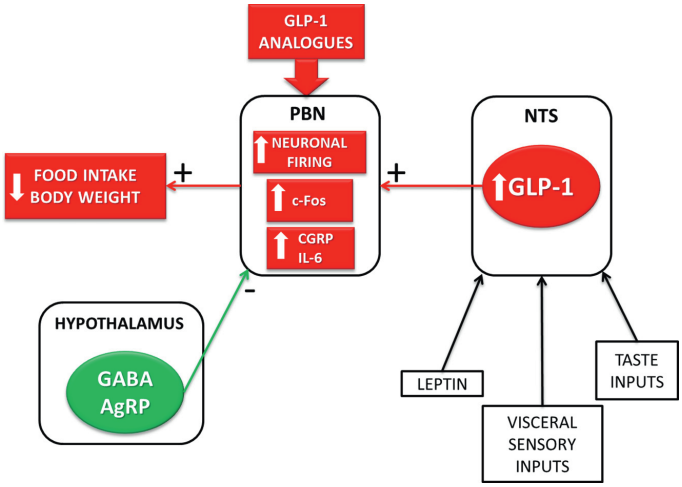

Figure 8. Graphical summary of results. Collectively, our data reveal the lPBN as a neural substrate for the feeding and body weight suppression effect of GLP-1 and identify the mechanisms involved. Elements of this novel energy balance relevant circuit identified in the current study are indicated in red. In contrast to the excitatory GLP-1 projections to the PBN, the projections from the hypothalamus (green) to the PBN provide inhibitory inputs, and their activation results in an orexigenic response.

Both taste and caloric value, processed in the PBN, may contribute to the PBN-relayed dopamine response. Activation of PBN neurons, akin to that observed here with Ex-4, can reduce the hedonic properties of food and inhibition of PBN neurons by microinjections of GABA-A receptor agonists into the PBN increases hedonic responses to oral sucrose (35).

The PBN is a heterogeneous nucleus with at least 12 distinct subnuclei and subdivisions of the PBN can be clearly differentiated based on their neuronal inputs and outputs (36). The gustatory afferents are represented in the medial subdivision, and the viscerosensory, the cardiovascular, and the respiratory functions in the lateral subdivision. In the current study, the decision to target the lPBN was based on the idea that caudally located GLP-1 neurons are likely to project to the viscerosensory lPBN rather than the mPBN, because inputs from the caudal viscerosensory NTS are segregated from the gustatory inputs from the rostral NTS to the mPBN. However, the segregation of inputs does not prevent some cross-communication, because even the gustatory PBN displays sensitivity to the metabolic status, and the lPBN, especially the dorsal part, is activated by noncaloric gustatory stimuli like saccharine (37). This may be the reason why both caloric food (chocolate and chow) and noncaloric saccharine consumption were reduced in the current study. Our study shows that activation of the GLP-1R can suppress intake of a sweet noncaloric solution.

The PBN plays a critical role in relaying visceral signals to the forebrain (38). The parabrachial-associated effect of a gut/brain peptide to reduce food intake, demonstrated here, fits well with previous studies showing that lPBN lesion impairs cholecystikinin (CCK)- and amylin-induced food intake suppression and attenuates the c-Fos activation normally expected from CCK and amylin action in the central nucleus of the amygdala (39, 40). Here, we show a direct effect of GLP-1R in the PBN. This is different from the previous studies, performed with peripheral injections of CCK and amylin, which could not determine whether these peptides exert direct or indirect feeding effects at the lPBN.

Signaling at the central GLP-1R is necessary for hypophagia induced by satiety and metabolic signals, like CCK and leptin, that are recruited in health, but it is also a key mediator of hypophagia induced by aversive stimuli, like lithium chloride and lipopolysaccharide (41–43). Interestingly, the same sickness-associated hypophagic stimuli can activate PBN neurons, and this activation may be a necessary component of the feeding suppression they cause (44–46). These 2 components are tied together by data indicating that hindbrain selective blockade of GLP-1R prevents lipopolysaccharide-induced hypophagia (47). Thus, it is possible that the GLP-1 neuron projections to the PBN and the hypophagia resulting from lPBN GLP-1R activation are relevant relays for sickness-induced hypophagia and not only for homeostatic appetite during health, as discussed above.

PBN neurons project to the hypothalamus, limbic system, and other forebrain regions. In order to begin to understand the potential downstream circuitry activated by GLP-1R stimulation in the PBN, we determined gene expression levels for candidate genes recently shown to be key for appetite suppression in the PBN. We found that Calca, the gene that encodes CGRP, an anorexic peptide, was elevated by Ex-4 treatment. CGRP neurons, found exclusively in the lPBN, play a key role in appetite suppression (18, 48, 49). Optogenetic stimulation of these neurons suppresses food intake in fed and food-deprived rats. c-Fos studies indicate that these neurons are activated by satiety signals, like amylin and CCK, and also by illness inducing signals like lithium chloride and lipopolysaccharide. Here, we show that GLP-1R activation can also stim-

ulate CGRP gene expression. Our neuroanatomical and immunohistochemical data suggest that this effect could be exerted by direct inputs from GLP-1 releasing fibers onto the CGRP-producing cells, because we found that most NTS-originating GLP-1-producing fibers innervated CGRP neurons in the lPBN. The elevation of CGRP levels could contribute to the anorexic and weight-suppressing effect of GLP-1, because direct intra-lPBN injections of CGRP reduced both food intake and body weight gain. It is noteworthy that GLP-1R stimulation increased CGRP only in ad libitum-fed rats, a more physiological situation for endogenous GLP-1 release. The lack of effect of GLP-1R activation on CGRP in fasted rats may indicate that orexigenic signals abundant during fasting may inhibit GLP-1's ability to induce CGRP, thereby reducing its ability to suppress food intake. This result is in line with a previous report showing a nearly complete suppression of GLP-1 neuron activation by food deprivation (50). Taken together, the present and previous studies suggest that fasting can suppress both production/release of GLP-1 and GLP-1-stimulated downstream anorexic pathways.

IL-1 and IL-6 are key regulators of the inflammatory response (51, 52), but they may also play an important role in healthy animals to regulate metabolic function. Mice lacking IL-1R or IL-6 develop late-onset obesity as well as disturbed glucose metabolism (53–56). Recently, we identified IL-1β and IL-6 as key mediators of the appetite-suppressive effects of GLP-1 (28). The results of the current study complement the previous data by showing that GLP-1R activation can increase *IL6* gene expression not only in the hypothalamus and caudal brainstem but also in the PBN. The elevation of IL-6 in the PBN was detected irrespective of the feeding state of the animal, indicating that the relationship between Ex-4 and IL-6 is robust. Previous data link CGRP and IL-6 in the pituitary (57). Although our data do not directly examine this connection, CGRP may not be necessary for the Ex-4 induction of IL-6, because IL-6 levels were elevated in fasted rats, whereas CGRP remained unchanged. IL-1β mRNA was not altered in the PBN, in line with previous data showing that GLP-1 can induce IL-1β in the hypothalamus but not the caudal brainstem (28).

Collectively, this study reveals lPBN as a neural substrate for the feeding suppression effect of GLP-1 and identifies the mechanisms involved (Figure 8). This mechanism of action may be relevant to patients receiving Ex-4, or other GLP-1 analogues, given that these pharmaceuticals can cross the blood-brain barrier after peripheral application, and c-Fos results indicate that peripheral Ex-4 injections in rodents can activate the PBN (58).

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Biology of Sex Differences

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Sex and estrogens alter the action of glucagon-like peptide-1 on reward

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Abstract

Background: Feeding behavior is regulated through an intricate array of anorexic and orexigenic hormones acting on the central nervous system (CNS). Some of these hormones may have differential effects in males and females, effects potentially attributed to actions of gonadal steroids, especially estrogens. Central stimulation of the glucagon-like peptide-1 (GLP-1) receptors reduces feeding and food-reward behavior by acting on CNS regions important for the anorexic actions of estrogens. Thus, we propose that the action of GLP-1 on food intake and reward may differ between sexes.

Methods: Male and female rats were centrally injected with the GLP-1 analog exendin-4 (Ex4) in a non-deprived or food-restricted state; reward behavior was measured in a progressive ratio operant conditioning task. Intake of chow and palatable food were also measured. To determine if sex differences in the actions of Ex4 are due to interactions with estrogens, Ex4 treatment was preceded by treatment with a nonselective estrogen receptor-α (ERα) and ERβ or ERα-selective antagonist.

Results: Central injection of Ex4 revealed increased reward behavior suppression in females, compared to males, in the operant conditioning task. This increase was present in both non-deprived and food-restricted animals with larger differences in the fed state. Intake of chow and palatable food, after Ex4, were similar in males and females. Food reward, but not food intake, effect of Ex4 was attenuated by pretreatment with ER antagonist in both sexes, suggesting that estrogens may modulate effects of Ex4 in both sexes. Furthermore, central pretreatment with ERα-selective antagonist was sufficient to attenuate effects of Ex4 on reward.

Conclusions: Collectively, these data reveal that females display much higher sensitivity to the food reward impact of central GLP-1 receptor activation. Surprisingly, they also demonstrate that central ERα signaling is necessary for the actions of GLP-1 on food-reward behavior in both sexes.

Keywords: Glucagon-like peptide-1, GLP-1, Exendin-4, Reward, Estrogens, Sex, Obesity

Background

Sex is a basic biological variable that influences physiology and disease. Despite the overrepresentation of women in diseases resulting from disordered eating [1, 2], few preclinical studies have a clear focus to explore the neurobiology and physiology of food intake regulation in females. This sex gap is symptomatic of what is seen in preclinical medical research overall. According to a recent review of preclinical publications [3], nearly two out of three papers did not even report the sex of the animals used in the study. Nevertheless, physiology and pathophysiology of feeding behavior differ in male and female animals or humans. Recent data suggest that the female brain responds differently to different food intake regulating signals [4–6].

Glucagon-like peptide-1 (GLP-1) and its receptors have emerged as a successful therapeutic target for treatment of type-2 diabetes [7, 8]. The usage of GLP-1-based therapy is increasing at a fast pace; however, some aspects of GLP-1 function, especially pertaining to its role in the central nervous system (CNS), remain unexplored. Considering the increasing amount of patients receiving this treatment [9, 10], there is a certain urgency to develop a better understanding of the action of GLP-1 and its analogs on the CNS. Increasingly, more

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evidence is pointing to the CNS as a major target for GLP-1 [11]. In fact, derived from preproglucagon, GLP-1 is produced not only in the periphery (intestine, pancreas) but also in the CNS, primarily by neurons located in the nucleus of the solitary tract (NTS). Central injections of GLP-1 receptor (GLP-1R) agonists potently decrease food intake; this anorexic action has been ascribed to the hypothalamic and brainstem GLP-1R expressing targets [11–15]; the same CNS regions are implicated for the anorexic action of estrogens [16–19], providing neuroanatomical grounds for a potential interaction between GLP-1R activation, sex, and estrogens proposed here.

Food intake is regulated not only from brain regions traditionally recognized for their role in homeostatically driven feeding, like the hypothalamus and the hindbrain, but also from extra-homeostatic areas that control rewarding aspects of eating [20, 21]. Food reward is an important component in the development of overeating and obesity [22] and can be divided in two components, "liking" and "wanting," as previously described by Berridge et al. [23, 24]. Wanting is associated with incentive salience and motivation for a certain type of food and is often coupled to stimuli, which can trigger a desire to acquire or work for the rewarding component. Liking on the other hand is more commonly associated with palatability. Though these two systems are closely linked, they also have the ability to act independently of each other, and via separate neural pathways, to modulate reward [23]. The wanting component of food reward is closely linked to the mesolimbic neurocircuitry, especially the ventral tegmental area (VTA) and its dopaminergic projections to the nucleus accumbens (NAc) [25]. GLP-1Rs and GLP-1-carrying fibers can also be found in these areas of the brain, involved in motivated behavior and addiction [26]. GLP-1 can change reward behavior in males [27–31]. However, little is known about GLP-1-driven food-reward control in females. Here, we determined whether there are sex differences in the sensitivity of food reward, more specifically food motivation and consumption of palatable food, and chow intake behavior to central GLP-1R activation.

Methods

Animals

Female and male Sprague-Dawley rats (160–200 g at arrival, and mean body weights of 260 and 480 g for females and males, respectively, during testing, Charles River, Germany) were housed in a 12-h light/dark cycle, in individual cages with ad libitum access to chow and water, unless otherwise specified. Female rats presented with normal 4- to 5-day estrous cycles throughout the experimental testing. However, this study was not designed to analyze any potential impact of cycling. All testing was conducted during the light cycle. All studies were carried out with ethical permissions from the Animal Welfare Committee of the University of Gothenburg, in accordance with legal requirements of the European Community (Decree 86/609/EEC). All efforts were made to minimize suffering.

Drugs

Exendin-4, angiotensin II, ICI 182, 780 [32], and MPP dihydrochloride (MPPd) were purchased from Tocris (Bristol, UK). Ex4 and angiotensin II were dissolved in artificial cerebral spinal fluid (aCSF, vehicle for central injection), and ICI and MPPd were dissolved in aCSF with 10 and 20 % DMSO, respectively. All drugs were stored as aliquots at −20 °C.

Operant conditioning

The progressive ratio operant conditioning schedule is a procedure used to analyze motivated behavior (reward wanting, often compared to the human experience of craving) and measures the amount of work or effort that a subject is willing to put in to obtain a reward, in this case rewarding food in the form of sucrose, and it therefore mainly measures the wanting component of food reward. Operant conditioning training was conducted in rat conditioning chambers (Med-Associates, Georgia, VT, USA) as described previously [27, 33] in ad libitum fed rats. Rats were trained to press a lever for a 45-mg sucrose pellet. Training was conducted in four stages: rats were first trained on the fixed ratio 1 (FR1) schedule in 30-min sessions (single press on the active lever resulted in the delivery of one sucrose pellet), followed by FR3 and FR5 (3 and 5 presses per pellet, respectively), where a minimum of 30 responses per session on the active lever was required for advancement to the next schedule, concluding with progressive ratio conditioning until stable responding was achieved. Each progressive ratio session lasted for 1 h. Responding was considered stable when the number of pellets earned per session did not differ more than 15 % between three consecutive sessions. All operant response testing was performed after the responses stabilized. All drug injections and testing were performed during the light cycle, starting 2 h after lights-on as specified below, and testing commenced 20 min after drug injection. Injections were done in a counterbalanced, Latin square design, with at least 48 h separating each injection condition.

Brain cannulation

Due to variations in body weight between male and female subjects, in addition to differential fat pad location and size [34], along with the fact that Ex4 can cross the blood-brain barrier [35], direct brain administration was chosen for all substances tested in order to avoid the potential confounding effects from these variations. As a

result, our experiments are not relevant to effects solely mediated by peripheral GLP-1R. Rats were implanted with a guide cannula targeting the lateral ventricle [36] (26 gauge; Plastics One, Roanoke, VA): ±1.6 from the midline, 0.9 mm posterior to bregma, and 2.0 mm ventral to skull, with an injector aimed 4.0 mm ventral to the skull under ketamine anesthesia. The cannulas were attached to the skull with dental acrylic and jeweler's screws and closed with an obturator as described previously [37]. Placement was verified with the angiotensin II drinking test. Angiotensin II was injected at a dose of 20 ng in 2 μL of aCSF, and water intake was measured throughout the following 30 min. Rats who consumed a minimum of 5 mL of water within the measured time period were considered to have correctly positioned cannulas.

Effects of central Ex4 in *non-deprived* males and females on food-motivated behavior and food intake

Male and female rats were injected with Ex4 (0.1 or 0.3 μg/μL) or vehicle into the left ventricle (LV). Doses were previously shown to reduce food motivation in food-restricted male rats [27]. Twenty minutes after injection, the rats were placed in the operant conditioning chambers for a 1-h testing period. Testing was performed in a non-deprived state 2 h following the dark cycle period where rats had ad libitum access to chow. After testing, the rats were returned to their home cages and given access to palatable food—peanut butter. After 1 h, the amount of consumed peanut butter was measured and the rats received normal chow for the remainder of the 24-h period.

Effect of central Ex4 in overnight *food-restricted* males and females on food-motivated behavior and food intake

Rats were food-restricted overnight to approximately 50 % of their normal intake. On the following day, rats of each sex were injected with Ex4 (0.3 μg/μL) or vehicle and the operant conditioning test was performed 20 min after injection for 60 min. Peanut butter intake and chow intake were measured as described above.

Effects of central estrogen blockade on male and female food-motivated behavior after Ex4 treatment

Food-restricted male and female rats received injections of the estrogen receptor (ER) antagonist ICI (10 μg/μL) [38] or vehicle into the left ventricle (LV) prior to injection of Ex4 (0.3 μg/μL) or vehicle in this area. Food restriction and the higher Ex4 dose were chosen in order to induce reliable reward behavior reduction in both sexes. Operant conditioning, intake of peanut butter, and chow consumption were measured as described above.

Effects of central administration of the ERα-antagonist on male and female food-motivated behavior after Ex4 treatment

Overnight food-restricted rats were injected with 1.4 μg/μL [39] of the estrogen receptor-α (ERα) (Esr1)—antagonist MPPd or vehicle, accompanied by injection of 0.3 μg/μL Ex4 or vehicle, into the LV. Operant conditioning, intake of peanut butter, and chow consumption were measured as described above.

Statistical analysis

All the data are presented as mean ± standard error of the mean (SEM). Statistical significance was analyzed using Student's t test or one- and two-way ANOVA, when appropriate, with Sidak's or Holm-Sidak's post hoc tests (GraphPad Software, Inc., San Diego, CA). p values lower than 0.05 were considered statistically significant.

Results

Effect of central Ex4 treatment on reward and food intake in *non-deprived* males and females

To investigate potential differences in the effects of central Ex4 treatment between males and females, rats of both sexes were tested using a progressive ratio reinforcement schedule after administration of Ex4 (0.01 or 0.03 μg/μL) or vehicle. Two-way repeated measures ANOVA revealed a significant reduction in sucrose rewards earned, and lever presses for sucrose, at both doses of Ex4 in female, but not male, rats (Holm-Sidak's multiple comparisons test, $p < 0.0001$; Fig. 1a, c). The main effect of drug treatment was significant for both rewards earned and number of lever presses for rewards ($F_{(2,\ 108)} = 19.25$, $p < 0.0001$; $F_{(2,\ 108)} = 15.91$, $p < 0.0001$). The main effect of sex on rewards earned and lever presses was not significant ($F_{(1,\ 108)} = 1.790$, $p > 0.05$; $F_{(1,\ 108)} = 3.717$, $p > 0.05$, respectively). Importantly, there was a significant interaction between these two factors ($F_{(2,\ 108)} = 10.46$, $p < 0.0001$; $F_{(2,\ 108)} = 9.195$, $p < 0.001$). Data displayed as sucrose rewards earned and active lever presses in percent of vehicle value revealed significant differences for both parameters at both doses of Ex4 between males and females ($p < 0.05$, Fig. 1b; $p < 0.05$, Fig. 1d). Two-way ANOVA also revealed a significant effect of sex ($F_{(1,\ 108)} = 25.56$, $p < 0.0001$; $F_{(1,\ 108)} = 17.02$, $p < 0.0001$) and a significant interaction between sex and treatment ($F_{(2,108)} = 7.610$, $p < 0.001$; $F_{(2,108)} = 4.424$, $p < 0.05$).

Central GLP-1R stimulation led to a significant decrease in peanut butter consumption compared to controls in both sexes (two-way ANOVA, Holm-Sidak's multiple comparisons test, $p < 0.001$ and $p < 0.0001$ for females for the low and high dose of Ex4, respectively, and $p < 0.0001$ for males for both doses of Ex4; Fig. 1e). Two-way repeated measures ANOVA indicated that the

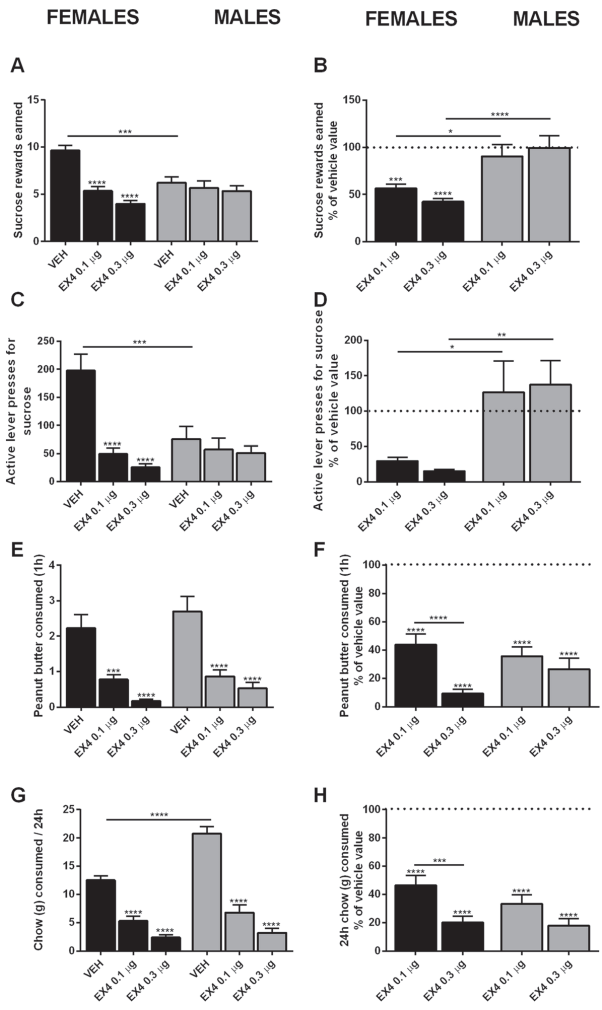

Fig. 1 In non-deprived animals, central GLP-1R stimulation specifically decreases food-motivated behavior in females but not males. Ex4 injection into the LV significantly reduced sucrose rewards earned (**a**), and active lever presses for reward (**c**) in female but not male subjects. Sucrose rewards (**b**) and active lever presses (**d**) were also significantly lower in females compared to males after Ex4 treatment, at both doses, analyzed as percent relative to vehicle value. Central GLP-1R agonist treatment significantly reduced peanut butter consumption in both males and females (**e**), with no significant differences between the two sexes (**f**). Chow consumption was reduced in males and females after Ex4 treatment (**g**); no significant differences were present between sexes at either dose of the agonist (**h**). Chow and peanut butter consumption are displayed in grams. Data are expressed as mean ± SEM. $n = 20$ (females) and 18 (males) per treatment group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$

main effect of sex on peanut butter consumption was not significant ($F_{(1,\ 108)} = 2.088$, $p > 0.1$). The effect of drug treatment, however, was significant ($F_{(2,\ 108)} = 36.38$, $p < 0.0001$) though there was not a significant interaction between these two factors ($F_{(2,\ 108)} = 0.2951$, $p > 0.1$). Data expressed as percent of vehicle value revealed a significant effect of drug treatment ($F_{(2,\ 108)} = 130.1$, $p < 0.0001$), but not the effect of sex ($F_{(1,\ 108)} = 0.4864$, $p > 0.1$; Fig. 1f) or interaction between these parameters ($F_{(2,\ 108)} = 3.026$, $p > 0.05$).

To investigate if 1 h peanut butter consumption is influenced by the experimental setup (the timing of the

test relative to injections or the fact that it is preceded by the operant task), an additional group of males received Ex4 injections (0.3 μg/μL) or vehicle, in a non-deprived state, and peanut butter consumption was measured 20 min after injection for 60 min (the same time point as operant conditioning testing was conducted). One-hour peanut butter consumption was significantly reduced in Ex4-treated male subjects compared to controls (Student's t test, $p < 0.05$, data not shown) at this time point. The size of the effect was comparable to the effect obtained when testing was conducted after PR, indicating that 1 h intake after PR testing was not affected by the experimental design.

In addition to peanut butter, Ex4 treatment led to a significant dose-dependent reduction in chow intake after 24 h, in both male and female rats (two-way ANOVA, Holm-Sidak's multiple comparisons test, $p < 0.0001$ for both doses of Ex4, in males and females; Fig. 1g). The main effect of drug treatment was significant (two-way ANOVA, $F_{(2,\ 108)} = 118.5$, $p < 0.0001$). Two-way repeated measures ANOVA also revealed a significant difference between males and females in both the main effect of sex ($F_{(1,\ 108)} = 21.01$, $p < 0.0001$) as well as interaction between sex and drug treatment ($F_{(2,\ 108)} = 9.590$, $p < 0.001$). Comparison of chow consumption relative to vehicle revealed a significant effect of treatment ($F_{(2,\ 108)} = 149.5$, $p < 0.0001$; Fig. 1h), but not sex ($F_{(1,\ 108)} = 1.698$, $p > 0.1$), and there was no significant interaction between these two factors ($F_{(2,\ 108)} = 1.051$, $p > 0.1$).

Effect of central Ex4 treatment on reward and food intake in *food-restricted* males and females

Due to the absence of effect of Ex4 on food-motivated behavior in non-food deprived males, an effect which has previously been established for fasted males, additional experiments in a food-restricted state were necessary to validate the experimental setup and to investigate potential differences in the action of Ex4 between sexes in different hunger states. Operant conditioning testing as well as food intake measurements were therefore conducted in overnight food restricted animals using only the higher concentration of Ex4. Under these conditions, Ex4 significantly decreased the amount of sucrose rewards earned in both males and females (Holm-Sidak's multiple comparisons test, $p < 0.0001$ and $p < 0.05$ for females and males, respectively; Fig. 2a). While the main effect of drug treatment was significant (two-way ANOVA, $F_{(1,\ 34)} = 38.41$, $p < 0.0001$), the main effect of sex was not ($F_{(1,\ 34)} = 1.219$, $p > 0.1$). Importantly, there was also a significant interaction between these two factors ($F_{(1,\ 34)} = 4.447$, $p < 0.05$). Though significant in both groups, females had a larger reduction in rewards earned than males (sucrose rewards earned as percent of vehicle value, $p < 0.01$; Fig. 2b). In the restricted condition, lever pressing for sucrose rewards reached significance in the female group after analysis using two-way ANOVA analysis ($p < 0.001$; Fig. 2c). Though the effect of treatment was significant ($F_{(1,\ 34)} = 21.66$, $p < 0.0001$), there was no significant effect of sex ($F_{(1,\ 34)} = 1.943$, $p > 0.1$) or interaction of sex and drug treatment ($F_{(1,\ 34)} = 2.725$, $p > 0.1$). Additionally, a significant difference was present between active lever press counts for restricted males and females (presses in percent of vehicle, $p < 0.01$; Fig. 2d).

In restricted animals, Ex4 treatment led to a significant reduction in 1 h peanut butter consumption in both sexes (two-way ANOVA, Holm-Sidak's multiple comparisons test, $p < 0.001$ for males and females; Fig. 2e). The main effect of drug treatment was significant (two-way ANOVA, $F_{(1,\ 34)} = 44.26$, $p < 0.0001$). The main effect of sex was non-significant ($F_{(1,\ 34)} = 1.545$, $p > 0.1$). There was no significant interaction between these two factors ($F_{(1,\ 34)} = 0.00183$, $p > 0.1$). There was, however, a difference in peanut butter consumption between male and female Ex4-treated groups when analyzed as percent peanut butter consumed relative to the amount consumed after vehicle injection ($p < 0.05$; Fig. 2f).

Ex4 treatment also led to a significant reduction in 24 h chow consumption, compared to controls, in both restricted males and females (two-way ANOVA, Holm-Sidak's multiple comparisons test, $p < 0.001$ and $p < 0.0001$ for females and males, respectively; Fig. 2g). Two-way ANOVA revealed significance in all measures, including the main effect of sex ($F_{(1,\ 34)} = 18.46$, $p < 0.001$), the main effect of drug treatment ($F_{(1,\ 34)} = 85.50$, $p < 0.0001$), and the interaction between these two measures ($F_{(1,\ 34)} = 10.14$, $p < 0.01$). However, no differences were found between Ex4-treated males and females when displayed as percent chow consumed compared to vehicle (Fig. 2h).

Effect of estrogen blockade on the actions of Ex4 on reward in food-restricted rats

To explore the impact of estrogens on the actions of Ex4, the estrogen antagonist ICI was administered centrally along with Ex4 into fasted male and female rats. Two-way repeated measures ANOVA revealed a significant difference in the main effect of sex ($F_{(3,\ 98)} = 14.71$, $p < 0.001$) and effect of drug treatment ($F_{(3,\ 98)} = 14.43$, $p < 0.0001$). A significant interaction between the two factors was also detected ($F_{(3,\ 98)} = 4.014$, $p < 0.01$). Pretreatment with ICI attenuated the Ex4-induced food reward suppression in female rats. After the combined treatment, the amount of sucrose rewards earned was therefore no longer statistically significant compared to controls (Fig. 3c). Interestingly, similar effects of the combined treatment on sucrose rewards earned were also present in males (Fig. 3a). For lever presses, two-way

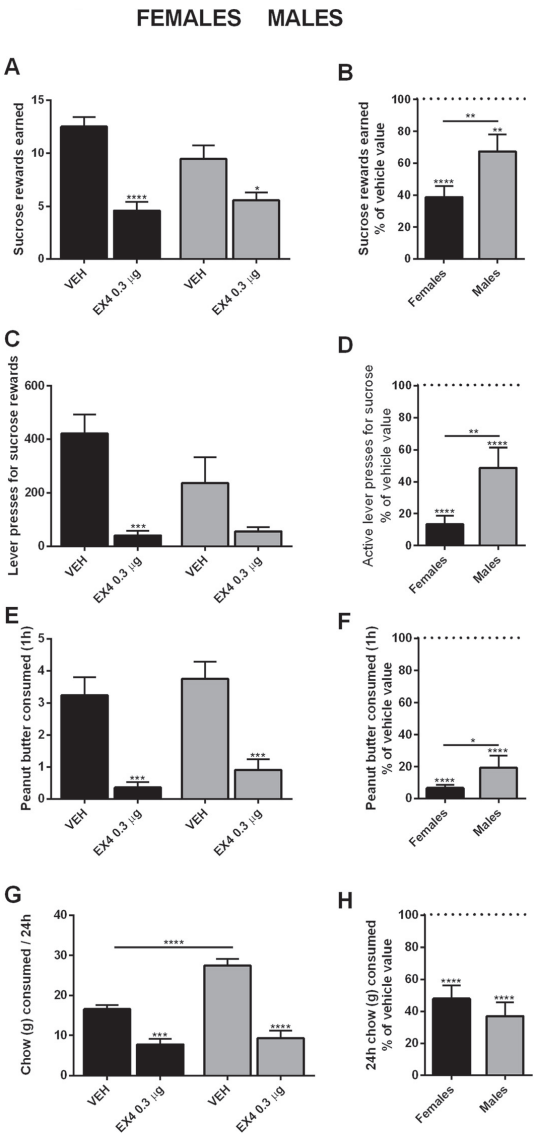

Fig. 2 Ex4 treatment in overnight food-restricted rats leads to a larger reduction in food-motivated behavior in females than males. After overnight food restriction, central Ex4 treatment reduced sucrose rewards earned in both males and females (**a**), an effect that was larger in females (**b**). Active lever presses were significantly reduced by Ex4 treatment in female subjects; a trend for reduction was present in male subjects but it did not reach statistical significance (**c**). Lever presses after Ex4 treatment in males and females analyzed as percent of vehicle treatment also revealed a significantly larger reduction in female subjects (**d**). Peanut butter consumption was significantly reduced in both sexes (**e**) with a slightly larger response to Ex4 in females (**f**). Twenty-four hour chow consumption was reduced in both males and females after GLP-1R agonist injection (**g**); no significant differences were found in chow intake between the two sexes (**h**). Chow and peanut butter consumption are displayed in grams. Data are expressed as mean ± SEM. $n = 10$ (females) and 9 (males) per treatment group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$

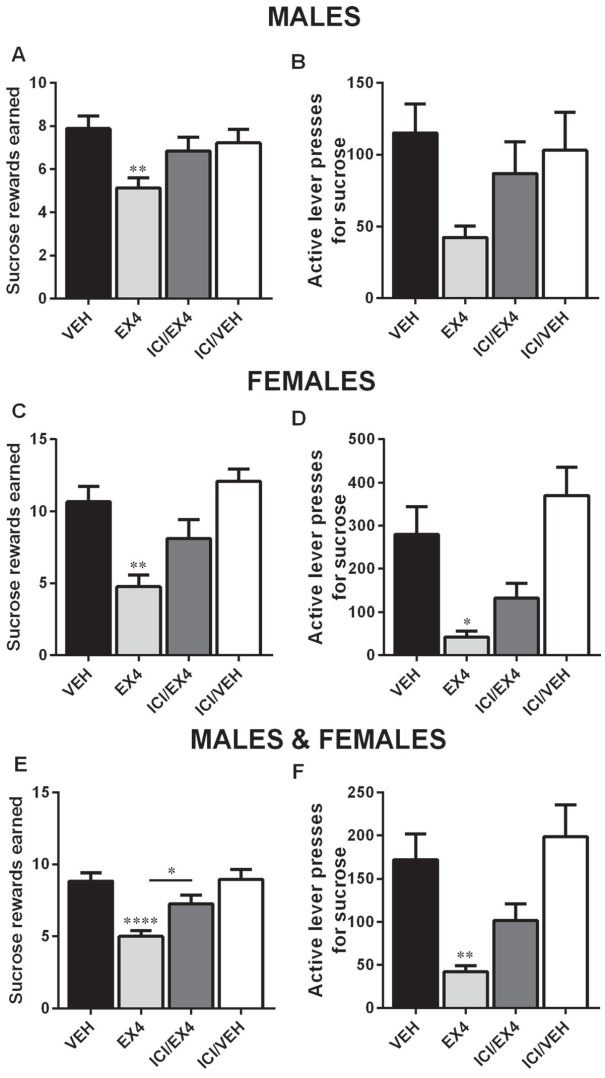

Fig. 3 Central estrogen blockade attenuates the effects of Ex4 on food-motivated behavior. Concurrent administration of the selective estrogen antagonist ICI and Ex4 attenuated the reduction in sucrose rewards earned in females (**c**) and males (**a**) compared to Ex4 treatment alone, although this effect did not reach significance. Reduction of active lever presses for sucrose was also attenuated in both sexes after ICI treatment (**b**, **d**). The number of sucrose rewards earned for males and females combined was reduced after central Ex4 injection (**e**); this effect was attenuated after ICI treatment. Combined results for males and females also demonstrate a significant reduction in lever presses for sucrose after central GLP-1R stimulation; attenuation by the ICI treatment did not reach statistical significance (**f**). Data are expressed as mean ± SEM. $n = 10$ (females) and 18 (males) per treatment group. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$

ANOVA indicated a significant effect of sex ($F_{(1,\ 98)} = 25.87$, $p < 0.0001$) and effect of drug treatment ($F_{(3,\ 98)} = 13.87$, $p < 0.0001$). Importantly, there was also a significant interaction between the two factors ($F_{(3,\ 98)} = 6.792$, $p < 0.001$). ICI treatment in females led to an attenuation of the reduction of active lever presses for sucrose after

central GLP-1R stimulation (Fig. 3d). In males, the reduced amount of active lever presses for sucrose did not reach statistical significance after Ex4 treatment, although interestingly, combination of Ex4 with the antagonist treatment did lead to an increase in the amount of lever presses compared to Ex4 treatment alone (Fig. 3b). When results of males and females are combined due to similar trend in response pattern, a significant difference in rewards earned (one-way ANOVA, $F_{(3,\ 102)} = 9.633$, $p < 0.05$; Fig. 3e), but not active lever presses for sucrose (Fig. 3f), between Ex4- and Ex4/ICI-treated rats is detected. ICI treatment alone did not have any significant effects on reward-related behavior in this experiment. One male rat and three female rats did not finish the study due to dislodged or blocked cannula.

Two-way repeated measures ANOVA indicated that the main effect of sex ($F_{(1,\ 63)} = 0.8913$, $p > 0.1$) was not significant. Drug treatment ($F_{(3,\ 63)} = 4.682$, $p < 0.01$) was significant. A significant interaction between these two factors was not found ($F_{(3,\ 63)} = 0.3939$, $p > 0.1$). Treatment with Ex4 alone, or in combination with the estrogen antagonist ICI, led to a significant reduction in peanut butter consumption after 1 h in females (one-way ANOVA, $F_{(3,\ 33)} = 4.242$, $p < 0.05$; Fig. 4c), but not in males ($F_{(3,\ 30)} = 1.142$, $p > 0.1$; Fig. 4a). Chow consumption was significantly different, both regarding the differences in the effect of sex ($F_{(1,\ 97)} = 17.73$, $p < 0.0001$) and the main effect of drug treatment ($F_{(3,\ 97)} = 1.197$, $p < 0.001$). A significant interaction between these two factors was not found ($F_{(3,\ 97)} = 0.6991$, $p > 0.1$). Twenty-four hour chow consumption was reduced after Ex4 treatment in both male and female subjects (one-way ANOVA, $F_{(3,\ 66)} = 4.139$, $p < 0.05$; Fig. 4b, $F_{(3,\ 31)} = 9.230$, $p < 0.001$; Fig. 4d, for males and females, respectively). In contrast to peanut butter consumption, ICI treatment attenuated Ex4-induced food intake reduction in females, but not in males, resulting in an increased amount of chow intake consumed during the 24-h period (Fig. 4b, d).

Effects of central administration of the ERα-antagonist MPPd on male and female food-motivated behavior after Ex4 treatment

For sucrose rewards earned, two-way ANOVA revealed a significant difference in the main effect of sex (Holm-Sidak's multiple comparisons test, $F_{(1,\ 56)} = 7.319$, $p < 0.01$) and effect of drug treatment ($F_{(3,\ 56)} = 13.98$, $p < 0.0001$). A significant interaction between these two factors was not found ($F_{(3,\ 56)} = 0.5209$, $p > 0.1$). As shown previously, Ex4 significantly decreased the amount of sucrose rewards earned in both male and female rodents in the operant conditioning task (one-way ANOVA, $F_{(3,\ 28)} = 4.750$, $p < 0.5$; Fig. 5a, $F_{(3,\ 28)} = 10.04$, $p < 0.001$; Fig. 5c, for males and females, respectively). Coinciding treatment with MPPd attenuated the effects of Ex4 in both sexes (Fig. 5a, c). Two-way repeated measures ANOVA revealed that the main effect of sex ($F_{(1,\ 56)} = 9.483$, $p < 0.01$) and drug treatment ($F_{(3,\ 56)} = 10.36$, $p < 0.0001$) were significant for active lever presses. There was, however, no interaction between these two factors ($F_{(3,\ 56)} = 1.777$, $p > 0.1$). Active lever presses for sucrose were significantly decreased in females after Ex4 treatment (one-way ANOVA, $F_{(3,\ 28)} = 7.328$, $p < 0.01$; Fig. 5d), but not in males (Fig. 5b). The effects of Ex4 on lever presses were also attenuated after MPPd treatment in females and an increase in presses was also present in males, though this increase was not significant (Fig. 5d, b). Combined results for males and females showed a significant increase in sucrose rewards earned after MPPd/Ex4 treatment compared to Ex4 treatment alone (one-way ANOVA, $F_{(3,\ 60)} = 12.92$, $p < 0.05$; Fig. 5e). The attenuation of MPPd/Ex4 administration on active lever presses in males and females combined, however, did not reach significance (5F).

Two-way repeated measures ANOVA indicated that the main effect of sex was not significant ($F_{(1,\ 56)} = 1.413$, $p > 0.1$) for 1-h peanut butter consumption. The main effect of drug treatment was significant ($F_{(3,\ 56)} = 20.13$, $p < 0.0001$). A significant interaction between these two factors was not found ($F_{(3,\ 56)} = 0.7364$, $p > 0.1$). MPPd injection did not affect the action of Ex4 on peanut butter consumption which remained significantly reduced compared to vehicle in both males and females (one-way ANOVA, $F_{(3,\ 28)} = 10.21$, $p < 0.01$; Fig. 6a, $F_{(3,\ 28)} = 10.74$, $p < 0.001$; Fig. 6c, for males and females, respectively). Two-way repeated measures ANOVA revealed that the main effect of sex ($F_{(1,\ 56)} = 16.94$, $p < 0.001$) and drug treatment ($F_{(3,\ 56)} = 21.27$, $p < 0.0001$) were both significant for the 24-h food consumption. There was no interaction between these two factors ($F_{(3,\ 56)} = 0.3711$, $p > 0.1$). Furthermore, the 24-h food intake was also unaffected after combined Ex4 and MPPd treatment compared to Ex4 alone and was still significantly reduced compared to controls (one-way ANOVA, $F_{(3,\ 28)} = 9.905$, $p < 0.001$; Fig. 6b and $F_{(3,\ 28)} = 12.83$, $p < 0.0001$; Fig. 6d, for males and females, respectively). Two male and two female rats did not finish the study due to dislodged or blocked cannula.

Discussion

Preclinical research unraveling the neural basis of feeding behavior is almost exclusively conducted in male subjects, although recent evidence suggests females and males may regulate feeding behavior differently [4–6]. Likewise, reward-mediated behavior may be differentially regulated between men and women due to the impact of sex hormones [40, 41]. GLP-1 agonists have previously been shown to affect food intake and food-reward behavior [27, 29, 42, 43]. The current study provides evidence that Ex4, the long-lasting GLP-1 analog, may

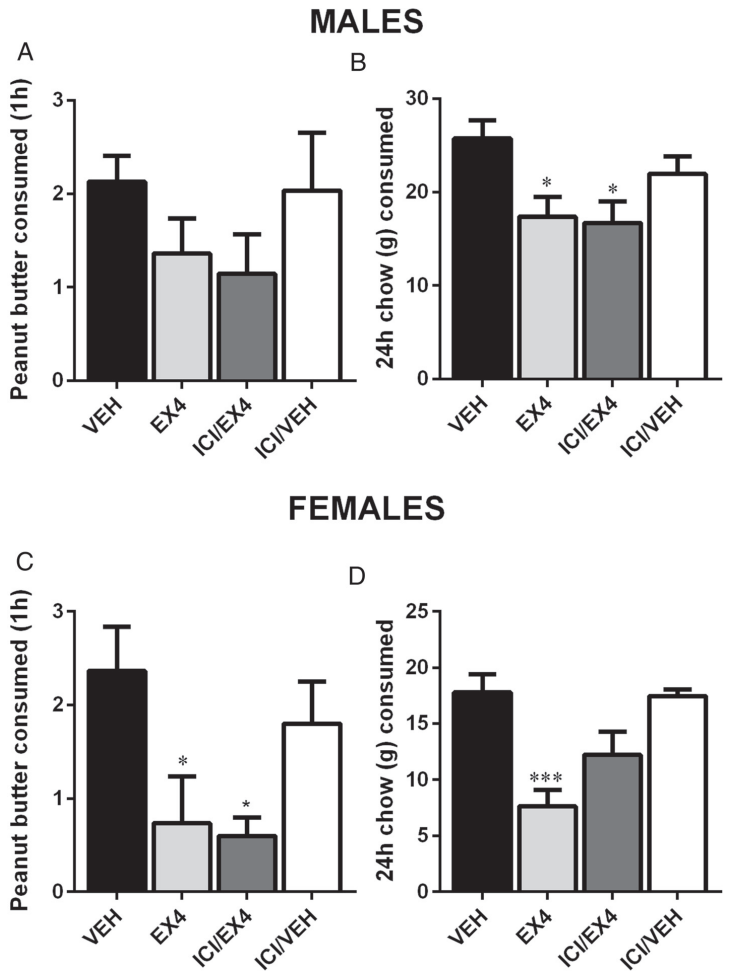

Fig. 4 Effect of central estrogen blockade on the actions of Ex4 on peanut butter consumption or chow intake is not divergent between the sexes. No significant changes in peanut butter consumption were observed in males (**a**), but a significant reduction in consumption was noted in females after injection of Ex4 or Ex4 and ICI (**c**). Ex4 reduced chow intake in both males and females (**b**, **d**). This reduction was attenuated by simultaneous ICI treatment only in females. Chow and peanut butter consumption are displayed in grams. Data are expressed as mean ± SEM. $n = 10$ (females) and 9 (males) per treatment group for peanut butter measurement and $n = 10$ (females) and 18 (males) per treatment group for chow intake. *$p < 0.05$, ***$p < 0.001$

have differential effects on food-reward in male and female subjects. Moreover, we demonstrate that signaling via central ER is necessary for the full impact of Ex4 on food reward. More specifically, signaling at the ERα may be crucial, since selective ERα-antagonist, MPPd, attenuated Ex4-induced food reward suppression.

Central GLP-1R agonist injection potently reduced food-motivated behavior in females and males in a sex-dependent manner in a progressive ratio operant conditioning task. The progressive ratio operant conditioning task, used here to analyze reward behavior, measures the motivation to obtain a food reward. Food-reward behavior is typically divided into two components: liking and wanting, where liking is typically associated with the immediate experience the moment a palatable food is consumed, while wanting is associated with reward seeking

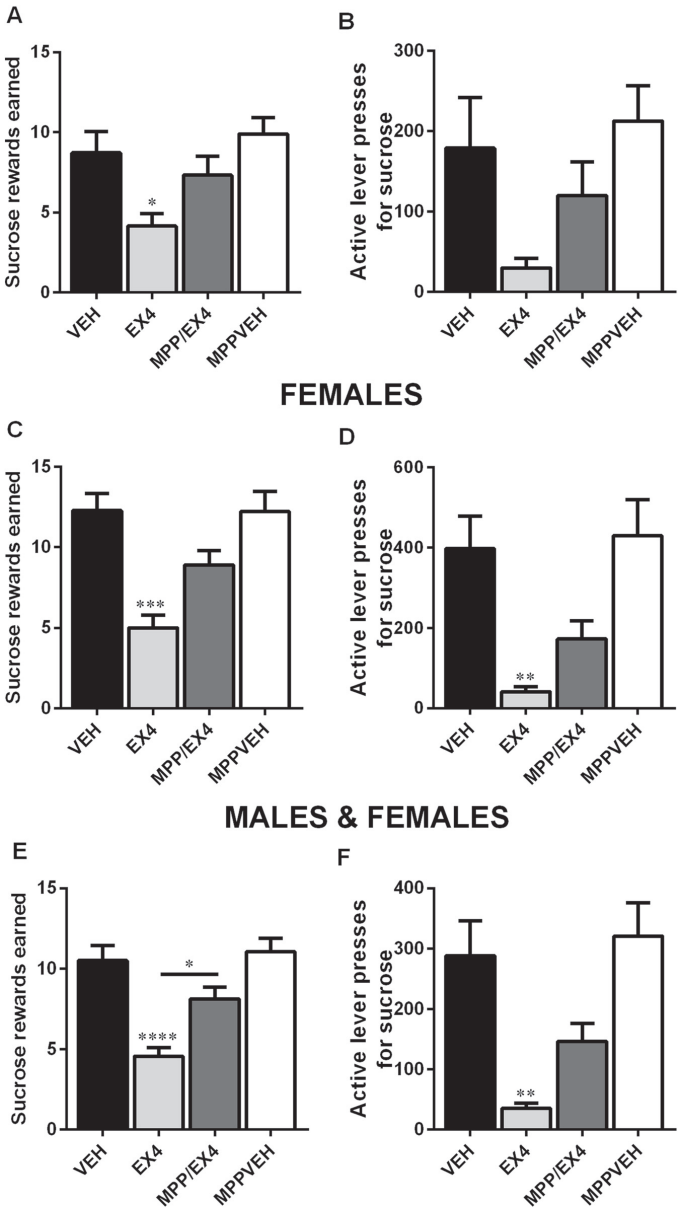

Fig. 5 (See legend on next page.)

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Fig. 5 Central injection of the ERα-antagonist MPPd attenuated the effects of Ex4 on food-motivated behavior. As expected, central administration of Ex4 led to a reduction in sucrose rewards earned in females (**c**) and males (**a**). Ex4 no longer significantly reduced sucrose rewards earned after administration of the specific ERα-antagonist MPPd. Additionally, Ex4 treatment reduced lever presses for sucrose earned in females (**d**) and produced a trend for reduction in males (**b**), an effect which was also attenuated by co-treatment with MPPd. Compilation of female and male data revealed a significant reduction in sucrose rewards earned (**e**) and active lever presses (**f**) after EX4 treatment that was attenuated after combined MPP/Ex4 treatment. Data are expressed as mean ± SEM. $n = 7–9$ for males and females. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$

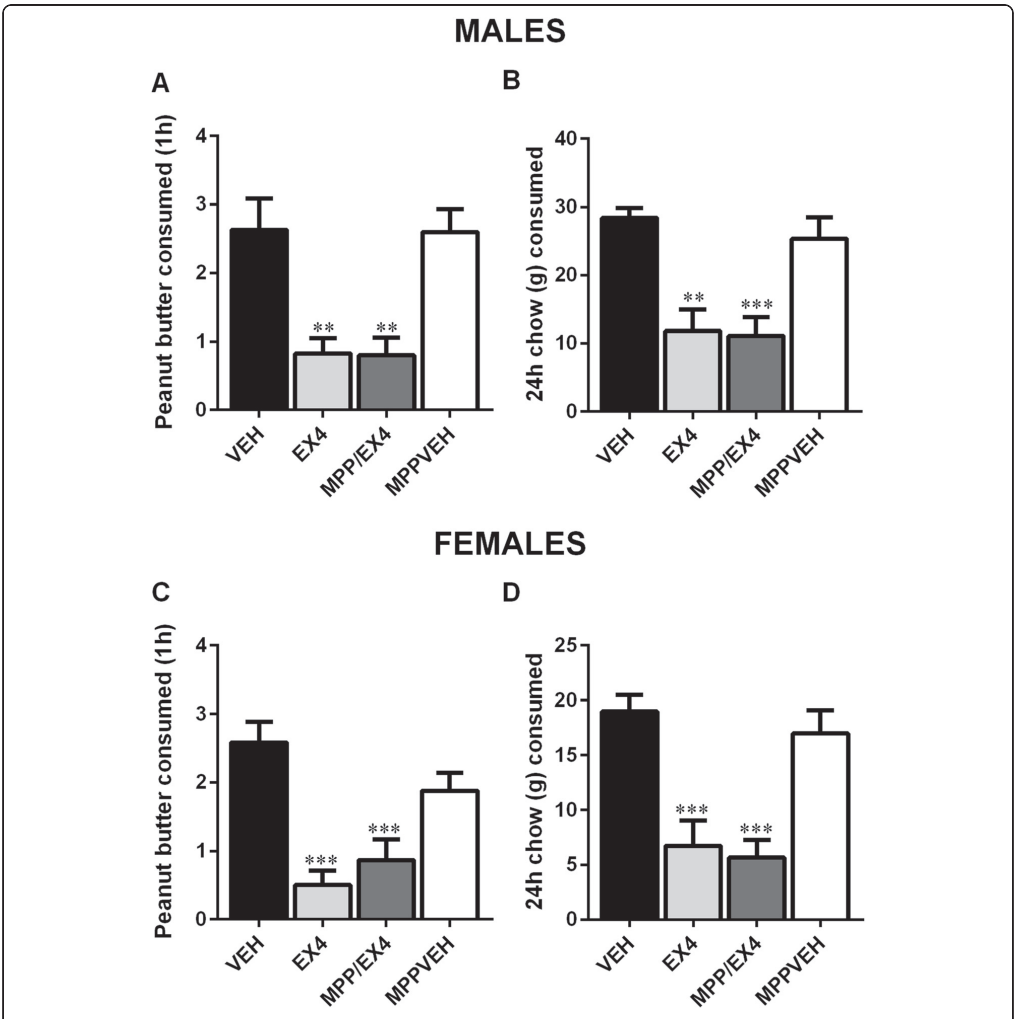

Fig. 6 MPPd treatment did not attenuate the effects of central Ex4 treatment on food intake. Central injection with the GLP-1R agonist Ex4 significantly reduced peanut butter intake and chow intake in males (**a**, **b**) and females (**c**, **d**). The reduction was still present after treatment with the ERα-antagonist MPPd. Chow and peanut butter consumption are displayed in grams. Data are expressed as mean ± SEM. $n = 7–9$ for males and females. **$p < 0.01$, ***$p < 0.001$

and an increased motivation to obtain rewarding foods [23]. Here, we show that the wanting component of the food-reward suppressing effects of Ex4 displays differential sensitivity in male and female rats. The actions of Ex4 on food reward were also accompanied by a reduction in palatable food intake in females and males compared to controls. However, peanut butter consumption response to Ex4 was not significantly different between male and female subjects. Progressive ratio test and palatable food intake may to some extent represent the two different aspects of food reward, where operant conditioning mainly measures wanting, while peanut butter intake better reflects the liking aspect of food reward. The differences in sensitivity to Ex4 treatment between sexes, therefore, seem to be specific to food-motivated behavior. Though, it is important to note that consumption of peanut butter is not a "pure" test of liking as it allows for ingesting ad libitum amounts of the palatable food; thus, an interaction with satiation signaling cannot be discounted.

Our data clearly indicate differences in the sensitivity to the food reward impact of Ex4 between females and males. Interestingly, only females showed a significant reduction in active lever presses, and sucrose rewards earned, in the non-food deprived state after Ex4 treatment. Yet, Ex4 has previously been shown to reduce food-motivated behavior and reduce the intake of palatable foods in males [27, 44]. However, considering that the preceding studies were conducted in rats that were food-restricted during training, and typically also testing in the progressive ratio task, in contrast to this experiment, the absence of reward reduction in males may indicate that Ex4 effect on food reward may be dependent on feeding state. A recent report indicates that a single overnight fast can attenuate GLP-1's suppressive effect on food intake. [45]. Here, we show similar results for the GLP-1 agonist, Ex4, as unrestricted access to chow resulted in a more potent reduction in chow intake after GLP-1R agonist treatment. For reward behavior, the interaction with fasting differs: overnight food restriction led to a significant reduction in sucrose rewards earned in male subjects after Ex4 administration. This effect was still less potent than in females, further indicating a higher sensitivity to Ex4 in female subjects. Baseline measures for reward slightly differed between sexes, with females receiving a larger quantity of rewards after vehicle treatment. The differential baseline values may cause some difficulty when comparing the effects of Ex4 in each sex since the higher baseline values displayed in females may make small reductions in reward caused by the drug more apparent. However, Ex4 treatment still reduced sucrose rewards earned in the food-restricted females to a larger extent than in food-restricted males, even though here the baseline values of rewards earned are not significantly different between the sexes. This further supports the hypothesis that females are more greatly affected by Ex4 treatment than their male counterparts.

Sex differences in reward were not accompanied by differences in overall food intake, indicating a potential selectivity of the sex differences to reward. The clear impact of Ex4 treatment on food intake suggests that the drug was in fact also effective in males, as expected based on previous studies with this agonist. However, the long-term interaction of estrogens with GLP-1 in women, including potential resulting sex differences in body weight, requires further investigation. Our results clearly suggest that when food availability is limited by the amount of work/effort the animal has to put in to obtain the food, Ex4 is much more efficient at reducing this type of behavior in females. Since food is rarely freely available to animals in nature, this effort-based eating behavior is likely very relevant to natural eating behavior. Surprisingly, only a few studies have looked at potential differences in food reward regulation between sexes in humans, though a recent study revealed differential connectivity in areas of the brain regulating reward between men and women which may indicate that food-reward behavior is differently regulated between sexes [46]. Recently, several studies investigated the effects of GLP-1R agonists on food intake and reward, and activation of intake or reward-controlling CNS areas in humans [47–49]; however, sex or gender interaction was not the focus, and therefore, sex was either not reported or the effect of sex not analyzed.

While the ability of estrogens to increase the potency of Ex4 in both sexes is unexpected, it is in line with one previous study indicating that co-administration of a GLP-1 and estrogen agonist in a conjugated form, that activates cells expressing estrogen receptors and GLP-1R, or GLP-1R alone, reduces food intake and body weight at doses of the hormones that are ineffective alone [50]. Importantly, the reduction was comparable in both sexes, suggesting that females and males benefit from the enhancing effects of estrogens on GLP-1 action. These data combined with current findings suggest that estrogens' signaling is sufficient to enhance food intake in both sexes but does not seem necessary for the anorexic effect of Ex4.

Estrogen receptors and GLP-1R are co-localized in areas involved in reward behavior regulation such as the VTA and the NAc [26, 51]. To our knowledge, little is known about the role of estrogens in food reward, though estradiol treatment in ovariectomized females was previously shown to reduce the intake of palatable, high-fat/high-sugar foods, in addition to reducing intake of standard food [52]. The role of GLP-1R activation in reward, however, is well-supported by recent literature

[27, 29, 44], though studies investigating the food-reward-mediated effects of GLP-1 have previously been conducted exclusively in males [27, 31, 53, 54]. Nonetheless, estrogens have been shown to cause functional changes in GABAergic neurons in the VTA and dopaminergic terminals within the striatum [55, 56], and estrogen treatment enhances striatal dopamine release [55, 57–61]. Furthermore, estrogens induce increased dopaminergic sensitivity to cocaine in ovariectomized rats [61]. In addition, administration of estradiol during the follicular phase increases the subjective effects of amphetamine [62]. Levels of dopamine, its metabolites and synthesizing enzymes, and amphetamine-induced dopamine release vary as a function of the ovarian cycle stage [57, 63]. Women progress faster to cocaine dependence and display a higher incidence of relapse; female rats also display a higher preference for cocaine rewards, potentially due to the effects of estrogens [40, 41]. In contrast, gonadectomy and sex hormone treatments in males do not have a large impact on cocaine-reinforced behaviors [64, 65]. Thus, while the food reward impact of estrogens is unknown, the literature at least suggests its impact on drug reward. Past studies focus on the rewarding effects of drugs of abuse where estrogens mainly enhance reward in contrast to the current results where estrogens reduced food reward by enhancing effects exerted by GLP-1. Interestingly, it has previously been reported that women report higher levels of craving to rewarding foods, such as chocolate; these cravings were reported to be most persistent during the periods of the menstrual cycle characterized by low circulating levels of estrogens [66] indicating that estrogens may indeed reduce food reward, in contrast to their effects on drug reward.

Current experiments utilizing ICI, a nonselective ER antagonist, revealed that estrogens may increase the sensitivity to Ex4 effects on reward. Estrogens' effects on food intake may be due to its ability to modulate the activity of endogenous hormones [4, 5]. Thus, surprisingly, the impact of estrogens on the anorexic action in the case of insulin is opposite to that previously seen for leptin and currently revealed for Ex4. Collectively, these studies illustrate estrogen's ability to modulate feeding behavior by acting on appetite-regulating hormones. Therefore, we hypothesized that estrogens may also play a role in the differential sensitivity displayed by males and females after Ex4 treatment. As hypothesized, central treatment with the ERα (Esr1) and ERβ (Esr2) receptor antagonist, ICI, attenuated the food reward effects of Ex4 in females, but surprisingly, food-reward behavior in males was also attenuated by ER blockade. Past literature suggests that ERα is involved in homeostatic feeding and weight regulation, and deletion of ERα leads to obesity in both sexes in mice [67, 68]. In addition, administration of estradiol reduces food intake and prevents weight gain in wild-type, but not in ovariectomized transgenic mice lacking ERα, further indicating a role for ERα in homeostatic feeding [69, 70]. Previous studies have mostly failed to find a connection between ERβ and feeding behavior [69, 70]. In contrast, selective activation of ERβ receptor was even shown to increase cocaine-seeking behavior, an effect that was not seen after ERα receptor stimulation, indicating that ERβ may affect reward-related behavior [71]. Thus, ERβ was an unlikely interaction target. Consistent with this idea, the selective ERα-antagonist MPPd was sufficient to attenuate the effects of Ex4 on reward, in both males and females, indicating that signaling via the ERα may be necessary for Ex4 impact on food-reward behavior. Though it is important to note that while MPPd is classified as an ERα antagonist and has been shown to antagonize estrogen-regulated genes in vitro and attenuate estrous-related decreases in food intake [72, 73], it has also been suggested to have estrogen-like activity since it increased uterine weight in rodents and reduced food intake in OVX rats [73]. Our data do not eliminate the possible involvement of ERβ, or other ER, such as GPR30. It is also possible that other gonadal steroids may be behind these differences. Testosterone, for example, regulates insulin sensitivity [74, 75]. However, in contrast to ovariectomy, orchiectomy decreases daily food intake and body weight by decreasing meal frequency, an effect which can be reversed by testosterone treatment [76, 77]. Potential effects of testosterone on Ex4 actions on food reward are yet to be investigated. Importantly, substances other than sex steroids may also be involved.

Although females have higher estrogen levels than males, males also possess a certain level of circulating estrogens due to the conversion of testosterone to estrogen, by the enzyme aromatase, in the reproductive tract, bone, and adipose tissue [78]. Since premenopausal females have much higher levels of circulating estrogens, the increased impact of Ex4 in females shown here may result from these elevated levels of estrogens. However, since estrogens can enhance the action of GLP-1 on food intake in males [50] and blockade of ER signaling reduces the impact of GLP-1R activation on food reward in males, even low levels of estrogens in males may be sufficient to increase the sensitivity to GLP-1. Moreover, the estrogen synthesis enzyme aromatase is also present in the brain, mainly in neurons in the hypothalamus and limbic system [79]. In addition, the anorexigenic effects of estradiol have previously been attributed to its actions within the CNS as only central but not peripheral blockade with ICI increases food intake [38]. Thus, it is possible that the small amounts of estrogens synthesized by the male brain are also sufficient to play an important role in the reward effects of GLP-1. It is even possible

that brain-synthesized estrogens may function as a potential downstream mediator of GLP-1 effects, an idea ripe for future studies.

The current study provides novel evidence for the effects of the synthetic GLP-1R agonist Ex4 on food-motivated behavior in females and its interaction with central estrogen signaling, but it also has some limitations. Here, for reasons described above, only central Ex4 application was used in contrast to the peripheral administration utilized during clinical treatment with these agonists. However, both GLP-1 and Ex4 have been shown to cross the blood-brain barrier [35, 80], and feeding or reward impact of Ex4 is mediated by CNS GLP-1R, indicating that GLP-1 agonists are likely to result in sex differences even after peripheral injection. In addition, the potential food-reward suppressing effects of endogenously produced GLP-1 may differ from those found here for Ex4; thus, the results here are more directly relevant to the clinically used GLP-1 analog, and the relevance to endogenous GLP-1 should be a topic for future investigation.

Conclusions

These data indicate that there are sex differences in the sensitivity to the food reward effects of Ex4. Moreover, signaling through the ERα is essential for the reward-reducing impact of Ex4. Synthetic GLP-1 analogs are widely used in the clinic as a treatment for type-2 diabetes, and one was also recently approved as a supplement for weight-management [81]. Although these drugs are prescribed to individuals of both sexes, preclinical studies have almost exclusively been done in male subjects, a concern which is present throughout many scientific fields. Moreover, these drugs have the ability to cross the blood-brain barrier and exert the effects evaluated here through their actions on the CNS. Data presented here may indicate a higher sensitivity to these substances in women. If our findings will be replicated in a future clinical study, then physicians prescribing this medication may need to consider establishing alternative doses for female patients taking these drugs.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

KPS designed the study. JER, RHA, LLF, and KO carried out all experiments. JER and KPS performed data analysis and wrote the manuscript. All authors read and approved the final manuscript.

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IV

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ORIGINAL ARTICLE

Lateral hypothalamic GLP-1 receptors are critical for the control of food reinforcement, ingestive behavior and body weight

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Increased motivation for highly rewarding food is a major contributing factor to obesity. Most of the literature focuses on the mesolimbic nuclei as the core of reward behavior regulation. However, the lateral hypothalamus (LH) is also a key reward-control locus in the brain. Here we hypothesize that manipulating glucagon-like peptide-1 receptor (GLP-1R) activity selectively in the LH can profoundly affect food reward behavior, ultimately leading to obesity. Progressive ratio operant responding for sucrose was examined in male and female rats, following GLP-1R activation and pharmacological or genetic GLP-1R blockade in the LH. Ingestive behavior and metabolic parameters, as well as molecular and efferent targets, of the LH GLP-1R activation were also evaluated. Food motivation was reduced by activation of LH GLP-1R. Conversely, acute pharmacological blockade of LH GLP-1R increased food motivation but only in male rats. GLP-1R activation also induced a robust reduction in food intake and body weight. Chronic knockdown of LH GLP-1R induced by intraparenchymal delivery of an adeno-associated virus-short hairpin RNA construct was sufficient to markedly and persistently elevate ingestive behavior and body weight and ultimately resulted in a doubling of fat mass in males and females. Interestingly, increased food reinforcement was again found only in males. Our data identify the LH GLP-1R as an indispensable element of normal food reinforcement, food intake and body weight regulation. These findings also show, for we believe the first time, that brain GLP-1R manipulation can result in a robust and chronic body weight gain. The broader implications of these findings are that the LH differs between females and males in its ability to control motivated and ingestive behaviors.

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INTRODUCTION

Obesity and associated metabolic diseases are continuing to increase worldwide while safe and effective obesity treatments remain elusive. As food intake and body weight are controlled by the central nervous system (CNS), understanding the complex CNS mechanisms that regulate energy homeostasis is essential for the discovery of successful antiobesity therapeutics.

The lateral hypothalamus (LH) is historically known as a critical 'feeding center' and a powerful coordinator of the drive to eat, drink and move. The key role of this nucleus in feeding control is illustrated perhaps most robustly by a profound aphagia induced by bilateral lesions of the LH.[1–4] Conversely, stimulation of the LH induces eating, even in satiated rats.[5] This nucleus is also suggested to be the interface between the homeostatic and hedonic control of feeding behavior. Rats will eagerly press a lever to stimulate their LH, suggesting that LH activation itself is rewarding.[6–8]

Food can be a very potent rewarding stimulus. Food consumption and reward are controlled by partly divergent and partly overlapping brain nuclei: areas that respond to hormonal and metabolic signals that are derived from the periphery and elsewhere in the brain.[9,10] The mesolimbic neurocircuitry, especially the ventral tegmental area (VTA) and its dopaminergic projections to the nucleus accumbens (NAc), have garnered much of the attention on neural control of food reward.[11–13] However, the LH is in fact the most potent self-stimulation center in the brain, and given that hungry/food-restricted rats self-stimulate even more than fed rats, an interaction between the metabolic status and reward may require processing by this brain nucleus,[7,14] although it is also possible that stimulation of the median forebrain bundle, which connects the VTA to the NAc and runs through the LH, may contribute to the reinforcing effects of the intracranial self-stimulation studies.[15] The LH is innervated by the hindbrain glucagon-like peptide-1 (GLP-1) neurons and GLP-1 receptors (GLP-1R) are expressed in the LH.[16] A potential reward impact of this communication is largely unexplored.

GLP-1 is an anorexigenic peptide that is produced both in the peripheral L-cells of the gastrointestinal tract and in the brain, primarily by neurons located in the nucleus of the solitary tract (NTS).[16] These neurons project to widespread, but discrete, GLP-1R-expressing brain areas.[16] Analogs of GLP-1 are currently approved for treatment of type 2 diabetes and obesity.[17] Administration of GLP-1 or the GLP-1R agonist, exendin-4 (Ex4),

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into the CNS reduces food intake and body weight, actions historically attributed to the hypothalamic and brainstem GLP-1R-expressing targets.[18–21] More recently, central GLP-1 signaling has been linked to food (see Kanoski *et al.*[21] and Skibicka[22] for reviews) and drug reinforcement.[23–25] Despite the reliable and potent inhibitory effects of pharmacological activation of GLP-1R on feeding and body weight—paradoxically, pharmacological blockade of central GLP-1R, and especially knockout studies of whole-body or neuronal GLP-1R, produce only minor changes in feeding or body weight.[26–28] Collectively, this phenomenon has led to an ever-increasing view that GLP-1R is only sufficient, but not critical, for body weight control. Here, utilizing a knockdown model of LH GLP-1R, we wanted to challenge that view and propose that central, and specifically LH, GLP-1R activation is indispensable to control of ingestive and food reward behaviors and ultimately body fat mass. We further wanted to examine behavioral, molecular and efferent targets of LH GLP-1R activation. Moreover, conceivable sex differences, as well as potential sources of differential female responses, including estrous cycle, were assessed.

MATERIALS AND METHODS

Animals

Male and female Sprague-Dawley rats (5 weeks of age at arrival, Charles River, Sulzfeld, Germany) were housed in a 12-h light/dark cycle (light on at 0700 hours) in individual cages with *ad libitum* access to chow (Teklad Global 16% Protein Rodent Diet (2016), Envigo, Huntingdon, UK) and water, unless otherwise stated. All studies were carried out with ethical permissions from the Animal Welfare Committee of the University of Gothenburg, in accordance with legal requirements of the European Community (Decree 86/609/EEC). All efforts were made to minimize suffering.

Drugs

Ex4 and the GLP-1R antagonist exendin-9 (Ex9) were purchased from Tocris (Bristol, UK), dissolved in artificial cerebrospinal fluid (Tocris; used as vehicle) and stored as aliquots at − 20 °C.

Brain cannulation

Guide cannulas were implanted into the LH as previously described[29] (for details, see Supplementary Information). With the chosen coordinates, our manipulations/injections consistently reached the central, lateral and dorsal LH. Injections may have also reached the zona incerta; however, unlike the LH, this area does not express GLP-1R[16] (Supplementary Figure S1).

Operant conditioning

The operant conditioning procedure is used to assess the motivation to obtain a reward, in this case food reward in the form of a sucrose pellet (45 mg TestDiet, Richmond, IN, USA). Training and testing were conducted as described previously[30,31] and in Supplementary Information. All operant response testing was performed under the progressive ratio (PR) schedule.

Effects of pharmacological LH GLP-1R activation or blockade on food intake, body weight, locomotor activity and food-motivated behavior

To test the effects of GLP-1R activation, 11-week-old rats were injected with Ex4 (0.05 or 0.15 μg) or vehicle (artificial cerebrospinal fluid), and operant conditioning response was tested 20 min after injection. Additionally impact of the estrous cycle on LH GLP-1R activation was evaluated in a separate group of 8-week-old females (see Supplementary Information). For experiments examining the effect of GLP-1R blockade, 11-week-old rats were first fasted overnight, then given a chow meal for 20 min and injected with Ex9 (10 μg) immediately after the meal. This design was modified from Hayes *et al.*[32] and used to elicit endogenous GLP-1 release to a controlled meal. Rats were tested in the operant conditioning task 10 min after injection. Food seeking was assessed as the number of head pokes into the feeding chamber during the 60 min operant session. Chow intake was measured 1 and 24 h after the operant testing sessions. Each treatment was counterbalanced where each condition was separated by a 4-day period. Locomotor activity was measured using horizontal infrared beams in the operant chambers (Med-Associates, Georgia, VT, USA).

GLP-1R knockdown

To knockdown the expression of the GLP-1R in the LH, a short hairpin RNA (shRNA) targeting GLP-1R transcripts was used (for details, see Schimdt *et al.*[24]). Preliminary *in vitro* studies demonstrated ~88% knockdown of GLP-1R expression in a rat neuronal cell line transfected with this shRNA.[24] To knockdown GLP-1R expression *in vivo*, this shRNA sequence was cloned and packaged into an adeno-associated virus (AAV) (serotype 1; titer = 5.22e12) in collaboration with the Viral Core at the University of Pennsylvania. This construct was previously shown to reduce GLP-1R expression *in vivo* in the rat VTA by 50%.[24] A green fluorescent protein (GFP)-expressing AAV (titer = 5.0e12) was used as a control.

To determine the functional significance of endogenous GLP-1R signaling in the LH, rats were surgically implanted with LH-directed guide cannulae as described above. Once rats achieved stable sucrose-motivated behavior on PR (at 9 weeks of age), AAV-expressing GFP (AAV-GFP) or the GLP-1R shRNA (AAV-GLP-1R-shRNA) was infused bilaterally into the LH (0.5 μl per hemisphere during 5 min). Microinjectors were left in place for 10 min after infusion to allow for diffusion away from the injection site. Rats chosen for each treatment group were matched for body weight, food intake and food reinforcement parameters on PR.

Body weight and food intake were measured daily after AAV construct infusion, except for days where fasting or food restriction was applied. Motivation to self-administer sucrose was assessed using a PR schedule 3, 7 and 10 days after AAV injections. On day 7, rats were food restricted overnight to determine whether the contribution of LH GLP-1R signaling to food-motivated behavior is altered by fasting.

Three weeks after viral injections, two experiments were performed that required food restriction: fasting blood glucose measurements and subsequent oral glucose tolerance test (intraperitoneal glucose tolerance test; for details, see Supplementary Information), and a fasting/refeeding experiment.

Four weeks after AAV injection, adipose tissue was weighed and the brains were collected. Brains were dissected and the LH, NAc and NTS were collected to assess GLP-1R expression using quantitative real-time PCR (for details, see Supplementary Information). An LH-containing coronal section (10 μm) was collected from each brain to verify correct placement of AAV injections by visualizing GFP fluorescence (see Figure 4 for a representative placement).

A second smaller group of rats ($n = 20$, 10 males and 10 females, 13-week old) was included after the first study to determine whether there are sex differences in the effects of LH GLP-1R knockdown and to follow food intake and body weight daily without the interruptions for testing that required overnight restriction carried out in the first group of rats. In addition, another group of female rats ($n = 16$) was tested to further evaluate the necessity of LH GLP-1R in younger females whose body weight gain is more rapid compared with the 13-week-old females; these rats were 9 weeks at the time of AAV-shRNA infusion.

Viral tract tracing and in situ hybridization

Injections of cholera toxin subunit B and *in situ* hybridization were performed as previously described.[33] For detailed methods, see Supplementary Information.

Impact of Ex4 or feeding on LH gene expression

Hypothalamic neuronal cells, immortalized from four 8-week-old male mice were used to evaluate the impact of GLP-1R activation on LH neuropeptides in neurons. In order to determine whether the genes found to be altered in the immortalized hypothalamic cell line were also expressed in the LH *in vivo* and altered by ingestion of fat, chow or sucrose, rats were exposed to respective diets for 1 h per day for 1 week on an every other day schedule. Brains were collected, and the LH was dissected immediately after the last feeding session. Gene expression was then analyzed (see Supplementary Information and Supplementary Table for details). In each feeding group, there was one animal that ate < 0.5 g of food; these rats were eliminated from final gene analysis.

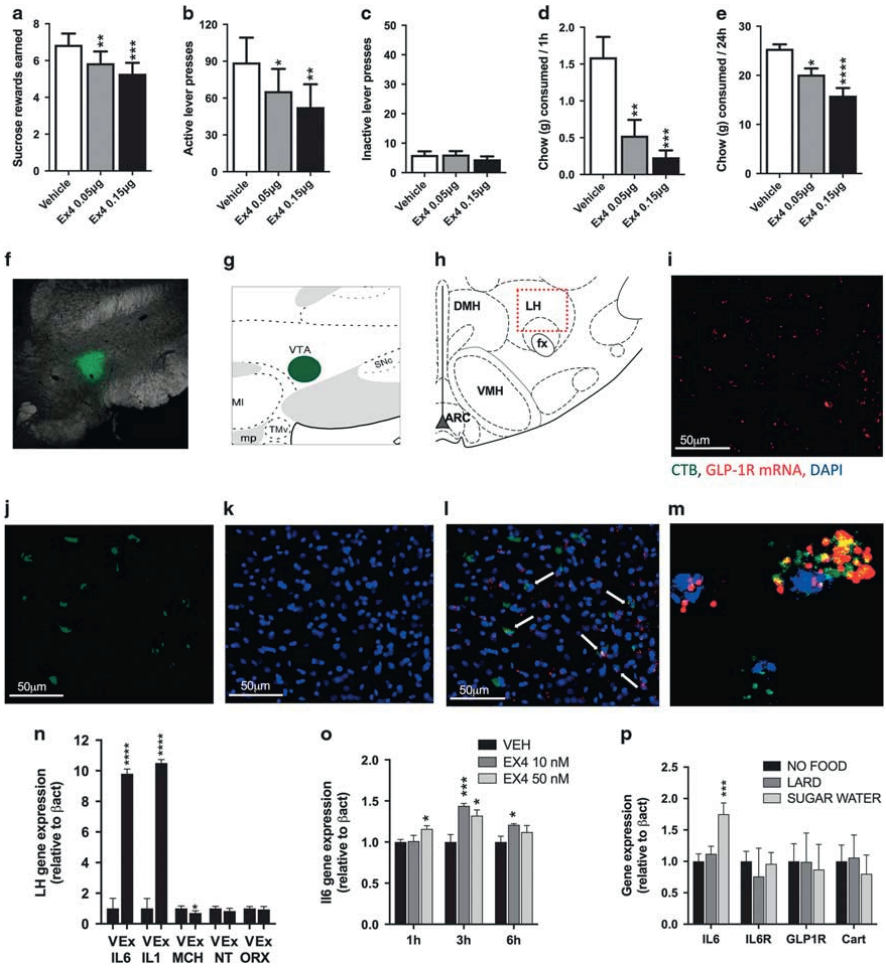


Figure 1. Behavioral, molecular and neuroanatomical consequences of glucagon-like peptide-1 receptor (GLP-1R) activation in the lateral hypothalamus (LH) in male rats. Activation of GLP-1R in the LH reduces food reinforcement and food intake. Intra-LH GLP-1 analogue, exendin-4 (Ex4), microinjection reduced the amount of sucrose rewards earned (**a**) and the number of lever presses for the rewards (**b**) in a progressive ratio schedule, without changing activity at the inactive lever (**c**) in male rats. Food ingestion is also affected, as illustrated by a potent reduction in 1 (**d**) and 24 h (**e**) chow intake. Data are expressed as mean ± s.e.m. $n = 14–15$ (male rats). Moreover, GLP-1R-expressing LH neurons were found to project to the mesolimbic ventral tegmental area (VTA), a likely neuroanatomical target area for reinforcement control exerted by LH GLP-1R. Representative images showing GLP-1R on LH neurons that project to the VTA. Cholera toxin subunit B (CTB) conjugated to AlexaFluor 488 was injected into the VTA (**f**, **g**). Back-labeled cells were present in the rostral LH region dorsal to the fornix (**h**) where the majority of the LH GLP-1R-containing neurons are localized. Images of GLP-1R *in situ* hybridization (red; **i**), back-labeled CTB-positive neurons from the VTA (green; **j**) and 4,6-diamidino-2-phenylindole (DAPI) nuclear counterstain (blue; **k**) at × 20 magnification. A merged image showing co-localization of GLP-1R and CTB in the LH (white arrows; **l**) and a confocal image taken at × 40 magnification of a double-labeled GLP-1R and CTB-positive neuron with DAPI counterstain (**m**). Intra-LH Ex4 infusion produced a marked increase in anorexic interleukins 1 (IL1) and 6 (IL6) and also reduced orexigenic LH neuropeptide expression (melanin-concentrated hormone (MCH)) in male rats (**n**). $n = 14–22$ (male rats). GLP-1R activation with Ex4 increased IL6 expression (**o**) in hypothalamic neuronal cell culture. In order to determine whether endogenous GLP-1 release also leads to similar changes within the LH, three groups of rats were allowed to eat chow, sugar or lard for 1 h. Lard meal did not alter any of the genes measured; however, a consumption of sucrose water increased LH IL6 expression (**p**). Data were normalized to the housekeeping gene beta-actin and are expressed as mean ± s.e.m.. $n = 7–9$ (male rats). ARC: arcuate nucleus of the hypothalamus; DMH: dorsomedial hypothalamus; VMH: ventromedial hypothalamus; NT: neurotensin; ORX: orexin; IL6R: IL6 receptor; Cart: cocaine- and amphetamine-related peptide. $^{*}P < 0.05$, $^{**}P < 0.01$,$^{***}P < 0.001$, $^{****}P < 0.0001$ compared with vehicle (artificial cerebrospinal fluid).

Figure 2. Behavioral and molecular consequences of glucagon-like peptide-1 receptor (GLP-1R) activation in the lateral hypothalamus (LH), as well as their interactions with the estrous cycle, in female rats. In females only the higher dose of exendin-4 (Ex4) reduced the amount of sucrose rewards earned (**a**) and resulted in a trend to reduce the number of lever presses for the rewards (**b**) in a progressive ratio schedule, without changing activity at the inactive lever (**c**). Food ingestion was also affected in female rats but only by the higher dose of Ex4, as illustrated by a reduction in 1 (**d**) and 24 h (**e**) chow intake ($n = 9$–37). LH GLP-1R activation in females has divergent behavioral and molecular impact in different estrus cycle phases. Intra-LH Ex4 reduced the effort to obtain a reward (**f**–**i**) in a sucrose-motivated progressive ratio task in females in the estrus phase (E) but not metestrus or diestrus (MD) phase of their cycle. Performance on the inactive lever or locomotor activity during the task was not altered by Ex4 in any of the cycle phases (**j**–**m**). Although Ex4 potently reduced ingestive behavior in all females irrespective of the cycle phase, the reduction was enhanced for females in E (**n**–**q**). Data are expressed as mean ± s.e.m. For each measured parameter, raw values and values calculated as the percentage of vehicle response are presented in order to minimize the impact of baseline differences across the two cycle groups. $n = 10$–11. Behavioral differences were mirrored by similarly divergent gene expression changes induced by intra-LH Ex4 application, where a potent induction of interleukins was only detected in females in E, and a reduction in orexin or neurotensin expression was only present in E (**r**). A reduced expression of melanin-concentrating hormone (MCH) was the only mRNA change preserved in MD phases. Data were normalized to the housekeeping gene beta-actin and are expressed as mean ± s.e.m. $n = 19$: vehicle in E, $n = 20$: Ex4 in E, $n = 8$: vehicle in MD, $n = 8$: Ex4 in MD. *$P < 0.05$, **$P < 0.01$,***$P < 0.001$, ****$P < 0.0001$.

Statistical analysis

All the data are presented as mean ± s.e.m. Statistical significance was analyzed using *t*-test or one- or two- way analysis of variance with Holm–Sidak's multiple comparison tests, when appropriate (GraphPad Software, San Diego, CA, USA). *P*-values < 0.05 were considered statistically significant.

Further information on methods is available in Supplementary Information.

RESULTS

Acute LH-targeted GLP-1R activation is sufficient to reduce food reinforcement and ingestive behavior and affect LH neuropeptide expression

Intra-LH Ex4 microinjection induced a robust reduction in food-motivated behavior, as indicated by the reduced number of sucrose rewards earned ($F_{(2,26)} = 12.59$; $P = 0.0001$; Figure 1a) and reduced number of lever presses emitted for the sucrose rewards ($F_{(2,26)} = 8.727$; $P = 0.0013$; Figure 1b), without changes in inactive lever pressing ($F_{(2,26)} = 0.7931$; $P = 0.4631$; Figure 1c) in male rats. Notably, *in situ* hybridization combined with viral tract tracing analyses revealed GLP-1R mRNA in ~55.3% of LH cells that project to the VTA, a region that has a well-established role in mediating food reward[11–13] (Figures 1f–m). The suppression of food intake was also very potent ($F_{(2,26)} = 11.10$; $P < 0.0005$; Figure 1d), with Ex4-injected rats consuming only 15% of the amount they ate while injected with vehicle. At 24 h, food intake ($F_{(2,26)} = 14.49$; $P < 0.0001$; Figure 1e) and body weight were still significantly suppressed (Supplementary Figure S2A) by intra-LH Ex4. Both the 0.05 and 0.15 μg doses significantly suppressed reinforcement, intake and body weight relative to intra-LH vehicle injection.

In order to determine which of the LH food and reward-regulating neurochemicals are affected by LH-targeted GLP-1R activation, Ex4 was microinjected into the LH of male rats, and gene expression of candidate genes was measured. Marked, 10-fold induction of anorexic interleukins 1 (IL1) and 6 (IL6) was found, along with a reduction in melanin-concentrating hormone (MCH) (Figure 1n). Both ILs were previously shown to mediate anorexic and weight loss effects of GLP-1 analogs.[34] We further showed that Ex4 induces IL6 in a hypothalamic neuronal cell culture, indicating that LH neurons could be one potential source of the Ex4-induced ILs (Figure 1o). Interestingly, we found that consumption of a sucrose solution, but not lard or chow meal, leads to LH IL6 induction (Figure 1p).

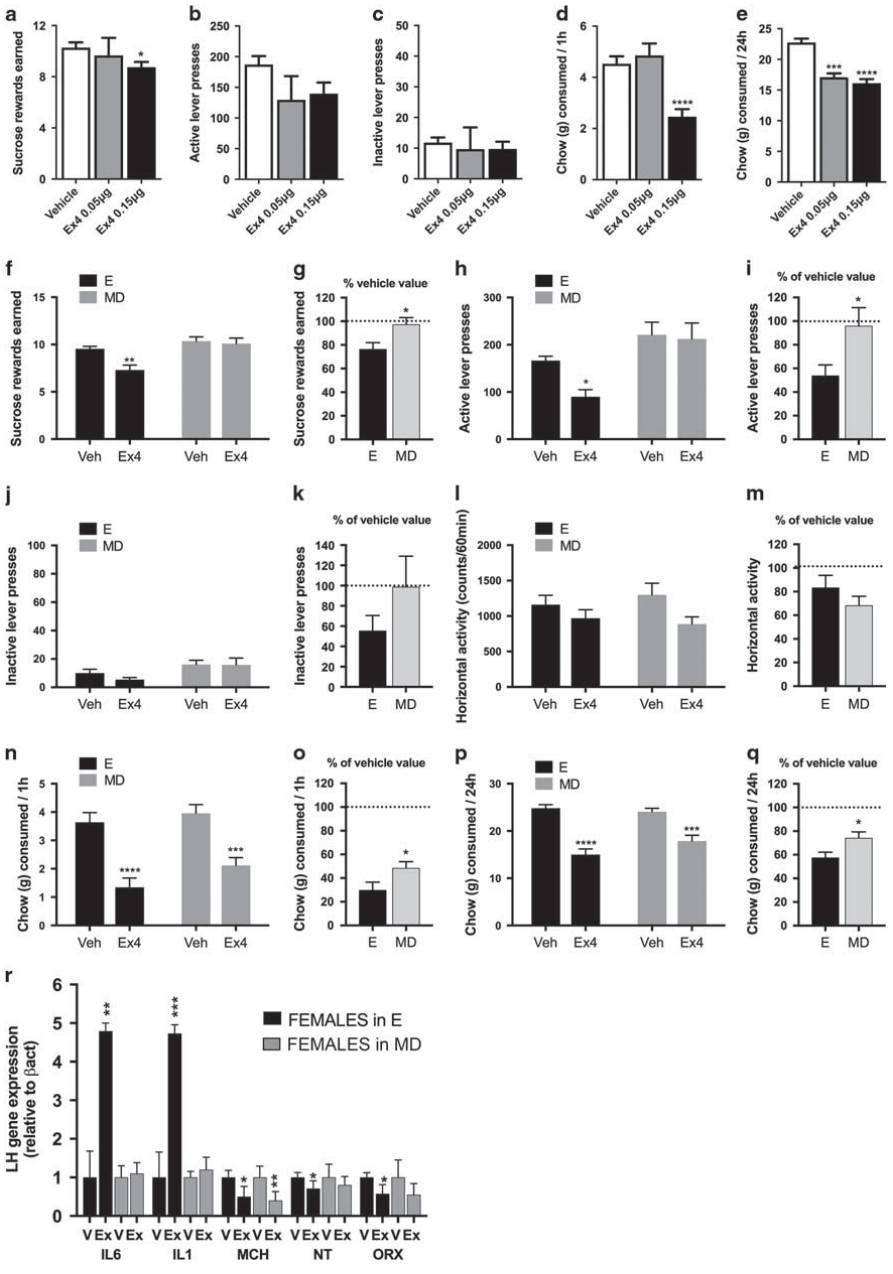
The same treatment produced a slightly less potent reinforcement and intake suppression in female rats, with the amount of sucrose rewards earned suppressed only by the higher Ex4 dose ($F_{(2,74)} = 2.81$; $P = 0.06$; Figure 2a). Active lever pressing did not reach significance ($F_{(2,74)} = 2.72$; $P = 0.07$; Figure 2b). One hour chow intake was also suppressed by the higher dose of Ex4 ($F_{(2,76)} = 15.43$; $P = 0.07$; Figure 2d), yet 24 h chow intake was reduced by both doses ($F_{(2,76)} = 29.51$; $P < 0.0001$; Figure 2e). Changes in 24 h weight gained were detected only for the higher dose (Supplementary Figure S2B). To test whether the estrous cycle stage affects behavioral impact of intra-LH Ex4 treatment, females were tested specifically in the estrus and metestrus/diestrus phases with the higher dose of Ex4 (0.15 μg), which was earlier found to be effective in females. Females in the estrus phase but not in metestrus/diestrus responded with reduced food reinforcement to the LH GLP-1R activation (rewards earned: $t_{19} = 2.7$, $P < 0.05$; active lever: $t_{19} = 2.3$, $P < 0.05$; Figures 2f–i). Although 1 and 24 h chow intake were reduced by the drug in all phases (Figures 2n and p), the drug effect was more potent in females in the estrus phase (1 h: $t_{19} = 2.2$, $P < 0.05$; 24 h: $t_{19} = 2.5$, $P < 0.05$; Figures 2o and q). The effect size seen was similar between males and females in estrus, yet significantly different when males and females in metestrus/diestrus were compared (Supplementary Figure S5). This cycle-dictated divergent behavioral impact of Ex4 was mirrored by Ex4-induced molecular changes in the LH, where a marked induction of anorexic IL1 and IL6 and conversely a reduction of the orexigenic neuropeptide, orexin, was found only in the estrus-phase females (Figure 2r). Reduction of MCH was the only drug-induced change detected in all phases of the cycle. In metestrus/diestrus, females show no changes in ILs compared with the 10-fold induction of each IL1 and IL6 in males (Supplementary Figure S6A). Molecular changes in the LH are similar between males and females in estrus, though males still display a more potent IL induction (Supplementary Figure S6B).

Acute LH-targeted GLP-1R blockade is sufficient to increase food reinforcement but not intake in male rats

Acute pharmacological blockade of LH GLP-1R by bilateral microinjection of Ex9 led to increased food rewards earned ($t_9 = 2.739$, $P < 0.05$, $t_{13} = 1.21$, $P = 0.701$, $t_{22} = 2.16$, $P < 0.05$ for males, females, and percentage of vehicle comparison between males and females, respectively), lever presses for sucrose ($t_9 = 3.59$, $P = 0.005$, $t_{13} = 1.344$, $P = 0.201$, $t_{22} = 2.44$, $P < 0.05$) and food-seeking behavior ($t_9 = 2.66$, $P < 0.05$, $t_{13} = 0.756$, $P = 0.463$, $t_{22} = 1.85$, $P = 0.07$) in male but not in female rats (Figures 3a–h) without changes in horizontal activity (Figures 3m and n). This treatment was, however, not sufficient to increase 1 h ($t_9 = 0.96$, $P = 0.36$, $t_{13} = 0.903$, $P = 0.383$, $t_{22} = 0.52$, $P = 0.61$) or 24 h food intake ($t_7 = 0.91$, $P = 0.39$, $t_{13} = 1.054$, $P = 0.311$, $t_{20} = 0.01$, $P = 0.98$) (Figures 3i–l). As previous experiments indicated that LH GLP-1R activation in females is only effective in the estrus phase, an additional group of rats was tested only in the estrus phase. However, even here female rats failed to increase food reinforcement or intake after bilateral LH microinjections of Ex9 (Supplementary Figure S7).

Chronic LH-targeted GLP-1R silencing leads to increased weight gain, food intake and food reinforcement

To determine whether endogenous GLP-1R signaling in the LH is necessary for control of body weight, food intake and motivation



for food reinforcement, AAV-GFP or AAV-GLP-1R-shRNA were administered bilaterally directly into the LH. This resulted in approximately 50% and 80% reduction (males/younger females and older females, respectively) in the GLP-1R transcript expression in rats infected with AAV-GLP-1R-shRNA when compared with controls (Figure 4n, Supplementary Figures S8A and B). This treatment was sufficient to disturb normal body weight and food intake regulation in 9-week-old male rats, as

AAV-GLP-1R-shRNA rats gained significantly more weight ($F_{(23,644)} = 10.69$; $P < 0.0001$, two-way repeated-measures analysis of variance) and ate more chow than control rats ($F_{(18,435)} = 4.583$; $P < 0.0001$) (Figures 4a and b). Elevated food intake and weight gain were detected in the third week after the treatment and the differences persisted until the termination of the study at 4 weeks. At that point, the weight of both fat pads measured, gonadal and inguinal white adipose tissue (GWAT: $t_{27} = 2.86$, $P < 0.01$ and IWAT: $t_{27} = 2.93$, $P < 0.01$), was also significantly higher (Figure 4d). LH GLP-1R knockdown also resulted in a much higher food intake and weight gain upon refeeding (1 h: $t_{27} = 3.62$, $P < 0.01$; 24 h: $t_{27} = 3.49$, $P < 0.01$; weight: $t_{27} = 3.07$, $P < 0.01$; Figure 4e), and a slightly higher blood glucose level during an intraperitoneal glucose tolerance test (area under the curve $t_{33} = 1.82$, $P = 0.07$; Figure 4c).

Knockdown of GLP-1R in the LH significantly increased food reinforcement in *ad libitum*-fed male rats, as indicated by increased number of rewards earned and more lever presses emitted during the PR operant test by rats infused with intra-LH AAV-GLP-1R-shRNA compared with AAV-GFP controls (Figures 4f and g), with the rates of pressing the active lever nearly doubling on both testing occasions. Food seeking was also potently elevated by the treatment (Figure 4h). These changes were not associated with nonspecific changes in locomotor activity (Figure 4i). An identical pattern of effects was detected in fasted rats (Figures 4j–m).

When comparing males and females, in the second experimental group, a significant increase in food intake was detected in male rats already by the end of the second week (cumulative intake: $F_{(20,\ 160)} = 9.95$ $P < 0.0001$; non-cumulative intake: $F_{(21,168)} = 4.48$ $P < 0.0001$; Figures 5a and d), and body weight gain reached significance 1 week after the onset of hyperphagia ($F_{(22,176)} = 7.57$ $P < 0.0001$; at day 19, Figure 5g). Interestingly, females did not show a consistent hyperphagia or weight gain (Figures 5b, e and h). The weight of the adipose tissue depots, examined 3 weeks after initiation of the knockdown of GLP-1R in the LH, doubled in males (GWAT: $t_7 = 2.2$, $P < 0.05$ and IWAT: $t_7 = 2.6$, $P < 0.05$, Figure 5j), but remained unchanged in females (GWAT: $t_7 = 0.4$, $P = 0.34$ and IWAT: $t_7 = 0.6$, $P = 0.3$, Figure 5k). Despite the large increase in fat mass and food intake, fasting blood glucose ($t_7 = 0.3$, $P = 0.4$) levels remained unchanged in male rats. Likewise, female fasting blood glucose levels were unaltered ($t_6 = 1.6$, $P = 0.08$; Figure 5m). In stark contrast to males, females did not show any changes in food-motivated or food-seeking behaviors (Supplementary Figure S9). Both males and females were adult (13 weeks of age) at the time of AAV infusion, and while males continued to gain weight during the entire 3-week period postinfusion, females at this age reached a weight plateau after the first 2 weeks of the study. To test whether the tapering growth rate in females is masking a potential impact of the GLP-1R knockdown, younger females (week 9, young adult) were tested. Surprisingly, these females displayed a drastically different response from the older females, where food intake (cumulative: $F_{(27,378)} = 15.18$, $P < 0.0001$, non-cumulative: $F_{(25,350)} = 3.244$, $P < 0.0001$, Figures 5c and f) and body weight ($F_{(26,364)} = 18.26$, $P < 0.0001$, Figure 5i) were massively impacted by the GLP-1R loss in the LH. The striking weight gain was likely due to fat gain, as at the end of the study the adipose tissue of these females nearly tripled (GWAT: $t_{13} = 5.9$, $P < 0.0001$ and IWAT: $t_{14} = 4.7$, $P < 0.001$, Figure 5l). Despite the profound impact of the LH GLP-1R knockdown on chow intake and adipose mass gain, we surprisingly found no effect on food reinforcement (Supplementary Figure S10). In an effort to not miss a potential late-developing effect in these young females or an effect only evident in the estrus phase, we additionally tested both parameters during week 4 and still found no effect (Supplementary Figure S10) of the LH GLP-1R knockdown on food motivation. Based on baseline daily feed efficiency calculations, we did not find any energy expenditure disturbances in either sex (Supplementary Figures S11A–C); however, we did find trends or a significant increase in feed efficiency after an overnight fast and upon re-feeding (Supplementary Figures S11D–F), indicating that LH GLP-1R may be necessary for energy expenditure regulation in some physiological contexts.

To test whether the reduction in GLP-1R in the LH elicits potentially adaptive counter-regulatory responses within the central GLP-1 system, GLP-1R expression was measured in the NAc (core and shell included), where GLP-1 also exerts food reinforcement-suppressing effects.[31,35] GLP-1R expression was increased more than twofold in the NAc of male rats in response to LH GLP-1R loss. Interestingly, this compensatory effect was not seen in any of the female groups (Figure 5o). This counter-regulation seems site-selective, as, for example, the NTS GLP-1R remained unaltered (Figure 5p). Finally, we also tested whether the hindbrain preproglucagon gene, a precursor of GLP-1, was altered in all GLP-1R knockdown groups irrespective of altered physiology or behavior. We found that expression of this gene was increased (Figure 5n), consistent with the brain GLP-1 system attempting to compensate for the loss of the LH node, indicating an interesting, and previously unexplored, capacity for adaptive compensatory plasticity within the brain GLP-1 system.

DISCUSSION

Although much is known about distinct populations of LH neurons, the metabolically relevant inputs to the LH are less clear. Here we show that the LH is a key brain area that mediates weight loss and the reward inhibitory effects of exogenous GLP-1R activation. Importantly, our data also demonstrate that the LH is a crucial site for motivated and ingestive behavior suppression driven by endogenously released GLP-1. With these results, LH emerges as one of the most important sites to date for endogenous GLP-1R activation, especially considering the size and robustness of the hyperphagia, weight gain and hypermotivation when GLP-1R in the LH are blocked and/or reduced. Although local LH GLP-1R activation was sufficient to reduce weight and reinforcement in both sexes, the effect of LH GLP-1R blockade was sex divergent, with the LH-GLP-1R signaling found to be crucial for motivated behavior control in males but disposable in females. Thus the female and male LH differs in its ability to control motivated behavior. The principal finding from these studies is that GLP-1R in the LH is required for normal body weight homeostasis and food reward control. This finding contrasts with conclusions drawn from studies carried out on GLP-1R knockout mice (for example, Finan *et al.*,[26] Scrocchi *et al.*[27] and Sisley *et al.*[28]), where little to no necessary contribution of CNS GLP-1R was demonstrated. Thus LH emerges as one of the most critical sites for the endogenous GLP-1 effect on energy balance and reinforcement.

In the context of food reward, the present data suggest that GLP-1, within the LH, inhibits motivation for palatable food. The effect of exogenous local LH Ex4 microinjection was fairly potent with a 30% reduction in the amount of effort the rats chose to expend for food reward. The VTA may be one of the neuroanatomical targets underlying this effect, as we found that more than half of the LH GLP-1R-expressing neurons project to the VTA. However, it is the impact of the endogenous GLP-1 in this area that is most striking, as when the GLP-1 signal is reduced acutely, male rats expend twice as much effort for the sucrose reward, indicating the crucial role of the endogenous GLP-1 in curbing food reward regulation in males. Compared with the LH, a smaller contribution was recently found with acute pharmacological blockade of GLP-1R in the dorsal part of the lateral septum[36] and with GLP-1R knockdown in the NTS.[37] In the ventral hippocampus, GLP-1R knockdown increases operant responding for food reward in non-restricted male rats (while not influencing

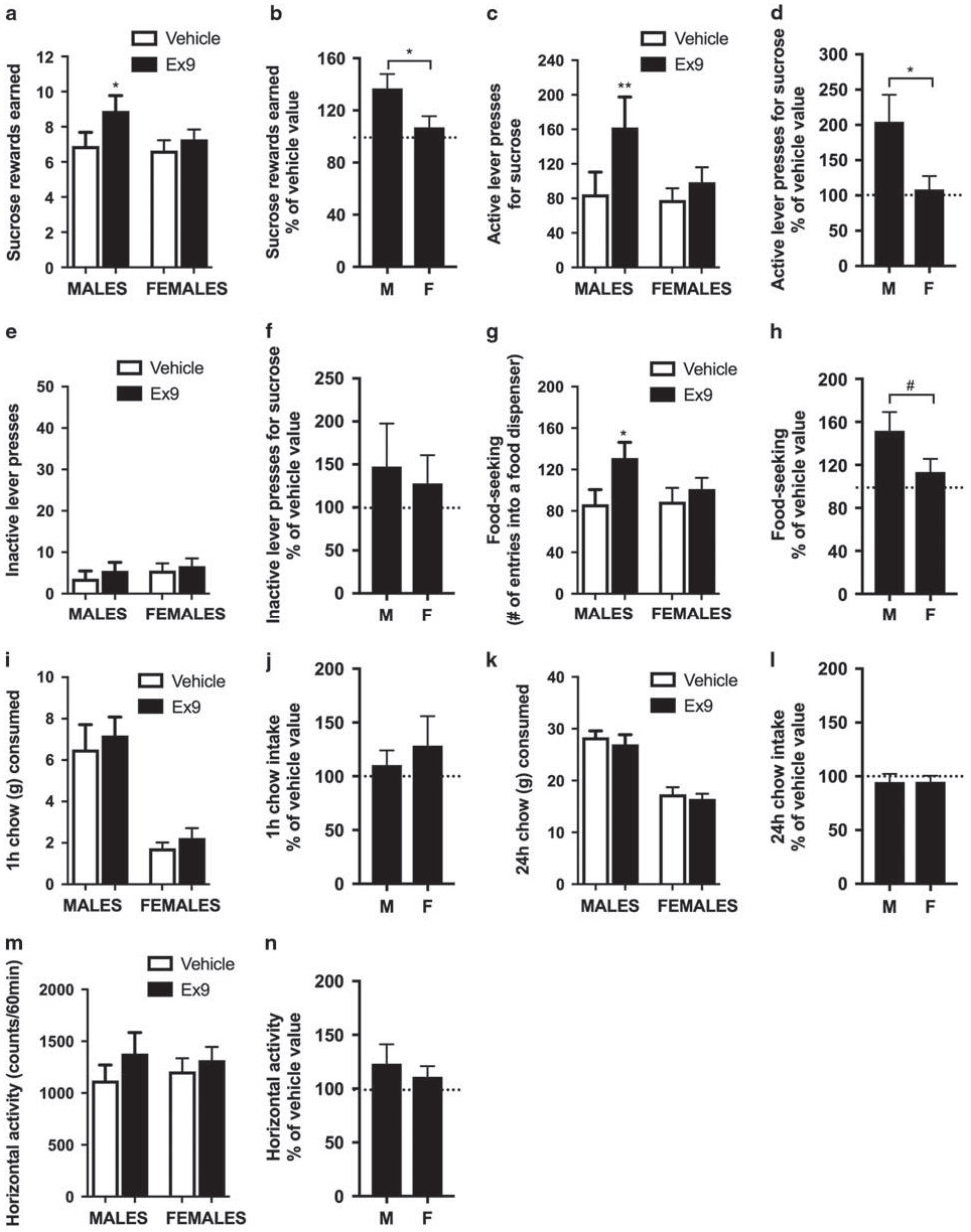
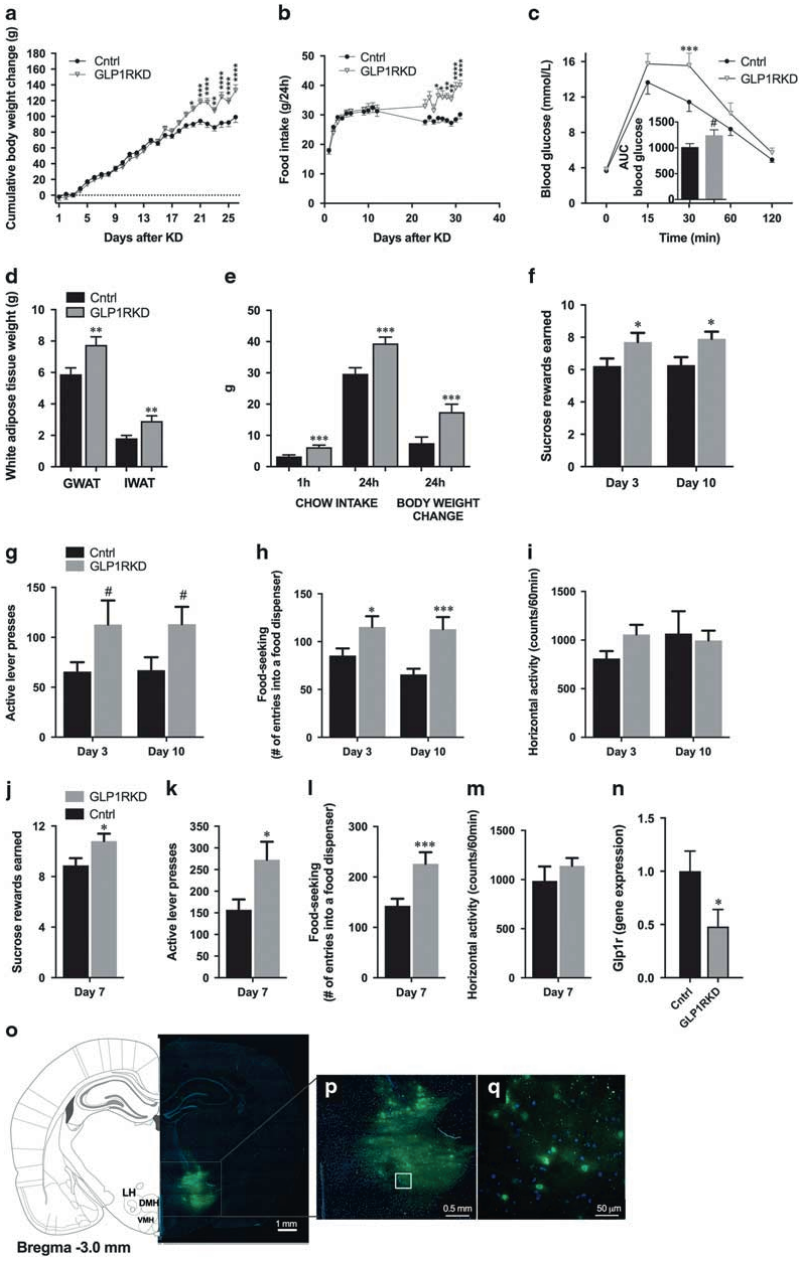


Figure 3. Acute pharmacological blockade of lateral hypothalamus (LH) glucagon-like peptide-1 receptor (GLP-1R) increases food reinforcement but not intake. Intra-LH microinjection of GLP-1R antagonist, Exendin 9 (Ex9; 10 μg) increased the amount of sucrose rewards earned (**a**, **b**) and the number of lever presses for the rewards (**c**, **d**) in a progressive ratio schedule, without changing activity at the inactive lever (**e**, **f**) in male rats. Similarly food-seeking (number of entries into a food dispenser) was significantly increased by this treatment in male rats (**g**, **h**). None of these parameters were altered in female rats. Food ingestion measured as 1 (**i**, **j**) and 24 h (**k**, **l**) chow intake were unaffected in either sex. Locomotor activity was also not significantly altered (**m**, **n**). Data are expressed as mean ± s.e.m. $n = 8$–10 (male rats: **m**) and $n = 14$ (female rats: **f**). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\#}P = 0.07$.



chow intake).[33] The impact of acute blockade of the GLP-1R in the LH is further supported by the enhanced food motivation detected after GLP-1R knockdown in the LH. It is of interest that the potently elevated food motivation is detected in both *ad libitum*-fed as well as food-restricted/re-fed male rats. This suggests that the detected food reward deregulation is robust and persists through different physiological/metabolic states.

The most striking result of GLP-1R knockdown in the LH was the persistent and potent hyperphagia: >50% increase in daily chow

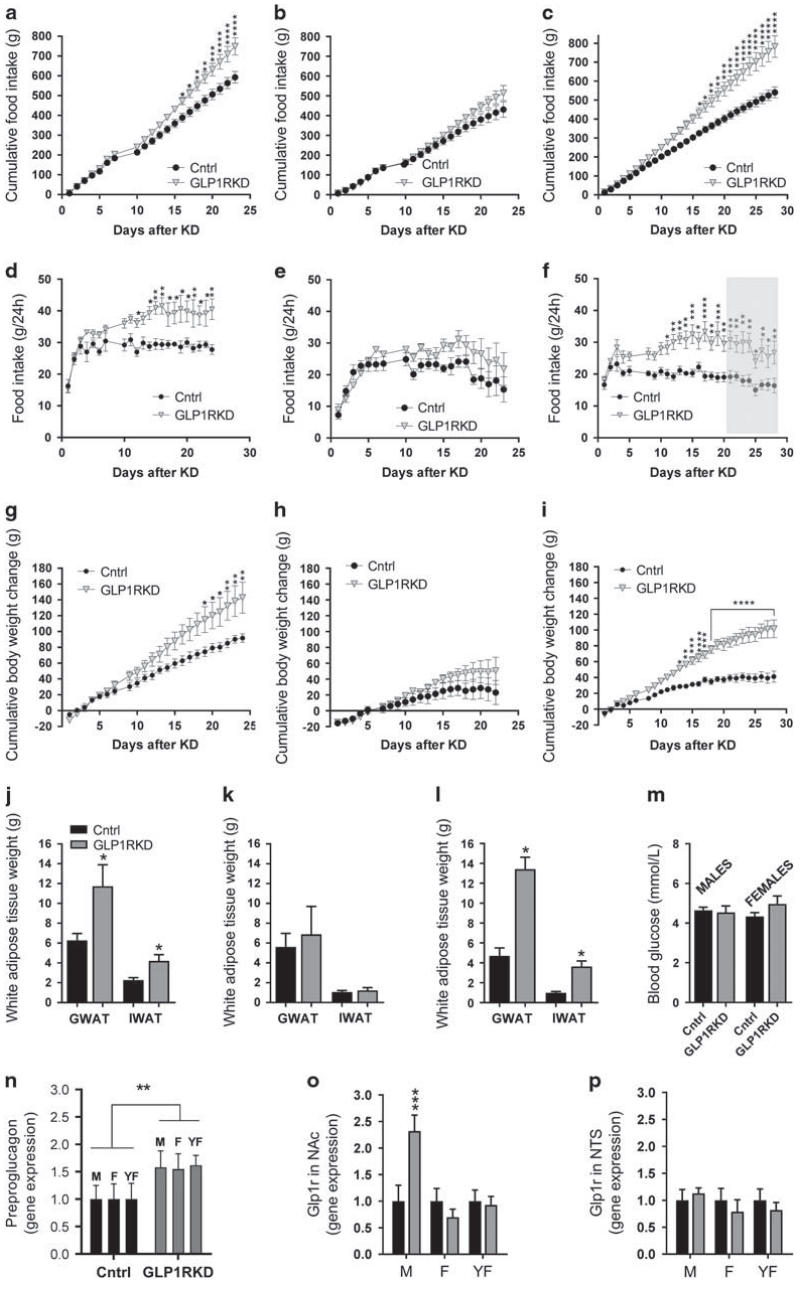
Figure 4. Glucagon-like peptide-1 receptor (GLP-1R) in the lateral hypothalamus (LH) are necessary for normal body weight, food intake and food reinforcement control. Decreased GLP-1R expression in the LH promoted weight gain (**a**) and hyperphagia (**b**), along with small impairments in glucose tolerance (**c**). Knockdown of GLP-1R in the LH led to increased fat mass in gonadal and inguinal adipose tissues (GWAT and IWAT, respectively; **d**). The hyperphagia and increased body weight gain persisted even after food deprivation/refeeding procedure (**e**). Data are expressed as mean ± s.e.m. $n = 14–15$ (male rats). Knockdown of GLP-1R in the LH resulted in an increased amount of sucrose rewards earned (**f**) and increased number of lever presses for the rewards (**g**) in a progressive ratio schedule. In addition, food-seeking (number of entries into a food dispenser) behavior was increased (**h**) without concurrent changes in locomotor activity (**i**). Similar potentiation of food reinforcement and seeking behaviors was detected in highly motivated (fasted) rats (**j–l**), again without significant changes in locomotor activity (**m**). Data are expressed as mean ± s.e.m. $n = 19–20$ (9-week-old male rats). Four weeks after adeno-associated virus (AAV) construct injections, GLP-1R expression was reduced by ~50% in the LH of rats treated with AAV-GLP-1R-shRNA (short hairpin RNA) compared with controls (**n**). Coronal brain section depicting LH-targeted infusions of control and AAV-GLP-1R-shRNA (**o**). Nuclear stain, DAPI (4,6-diamidino-2-phenylindole), is shown in blue. Panels (**p** and **q**) show green fluorescent protein-expressing cells in the LH. $^{*}P < 0.05$, $^{**}P < 0.01$,$^{***}P < 0.001$, $^{****}P < 0.0001$; $^{\#}P < 0.1$. DMH, dorsomedial hypothalamus; KD, knockdown; VMH, ventromedial hypothalamus.

consumption was detected, starting 2 weeks after the knockdown. This hyperphagia likely contributes to the massive increase in body fat, where the amount of fat detected in both fat depots examined (IWAT and GWAT) doubled in males and tripled in younger females. This is perhaps the most potent collection of effects on reward behavior and metabolism of a single nucleus and one receptor-encoding gene change detected to date. These data demonstrate the important contribution of the LH GLP-1R to homeostatic physiology and behavior. Interestingly, whole-body, or CNS-specific, GLP-1R knockout in a mouse did not result in sizable change in body weight, fat mass or hyperphagia.[26–28] There could be many reasons for this discrepancy; developmental compensation or species difference (mouse vs rat) are two possible explanations. Importantly, the combinations of generally weak effects of the pharmacological blockade of the GLP-1R along with little impact of the GLP-1R knockout led to a long-standing conclusion that, while GLP-1R analogs are an attractive pharmaceutical treatment option, the endogenous GLP-1 system is not necessary or critical for body weight and behavior regulation. Thus current results contradict this view and show that CNS GLP-1R, at least at the level of the LH, is necessary for normal body weight maintenance. Of note, we found that, while acute stimulation of GLP-1R in LH was highly effective at food intake and body weight gain suppression, acute blockade was not sufficient to alter chow intake or body weight. This may suggest that acutely other GLP-1R-expressing sites are able to compensate for the loss of GLP-1R signaling, compensation that proves insufficient when LH GLP-1R is silenced in a chronic manner. Although our data also indicate that, following chronic LH GLP-1R silencing, the brain attempts to compensate for the reduced anorexic signal in the LH by potently increasing GLP-1R expression in the NAc only in males and preproglucagon in the NTS of both sexes. This is clearly not sufficient to rescue the reward and metabolic phenotype in males, although we can hypothesize that simultaneous GLP-1R knockdown in both of these sites may produce an even more pronounced reward phenotype in males. We did not find any compensation in receptors from another GLP-1R-expressing site,[37–39] the NTS, in either sex or age.

Several studies revealed that peripheral hormones, ghrelin or leptin, affect food intake through actions on the LH orexin or neurotensin neurons.[40–43] Leptin and GLP-1 are known to interact, and both have been shown to reduce food intake, body fat, body weight and food reward, although the impact of reduced GLP-1R signaling in the LH observed in the current study is quite distinct from that reported after LH leptin receptor knockdown.[44] Leptin receptor knockdown does not change food intake or weight in chow-fed rats, and a high-fat challenge is necessary to bring out the disturbed metabolic phenotype, which is transient. This contrasts with the robust, and long-lasting, weight and intake elevation found here after GLP-1R knockdown. Importantly, leptin receptor knockdown did not yield changes in food-motivated behavior, while potent and lasting upregulation of food motivation was found here after GLP-1R knockdown, at least in males. Collectively, these data suggest that, while there may be some overlap in the cellular and molecular targets of leptin and GLP-1 in the LH, GLP-1 seems to stimulate a broader, at least functionally, fraction of LH cells. Interestingly, the VTA proved to be the key target site for food intake and reward effect of leptin.[45–47] For GLP-1, while many studies suggest that VTA GLP-1R are sufficient to inhibit food intake and motivation,[31,48] no data to date exist to show that they are necessary for these behaviors.

A number of genetically and functionally distinct cell populations reside in the LH,[49] although the neuropeptides or neurotransmitters affected by GLP-1R activation remain unknown. Acuna-Goycolea and van den Pol[50] reported a possible link between GLP-1 and the LH orexin neurons, where GLP-1 depolarizes orexin neurons and increases their spike frequency *ex vivo*. In our study, LH GLP-1R activation reduced orexin expression but only in females. Nevertheless, a more potent molecular effect of LH GLP-1R activation was to increase IL1 and IL6 in both males and females and also in a male-derived hypothalamic cell line. These ILs are not only clearly anorexic and weight reducing but are also already clearly indicated as mediators of anorexic and weight-loss effects of GLP-1.[34] Interestingly, our data also indicate that IL6 expression levels are increased by food intake, as sucrose meal, but not fat or chow meal, increased the levels of IL6 in the LH. Of note, the hypothalamic cell line was derived from males, thus it is possible, based on our *in vivo* results, that had this cell line been derived from females a smaller IL6 induction would be expected, as a 5-fold increase was found in the female LH compared with a 10-fold increase in IL6 mRNA in male LH. Expression of MCH, an orexigenic LH neuropeptide, was reduced by LH GLP-1R activation in both males and females, which could contribute to both the reinforcement and adiposity effects of LH GLP-1R activation.

Sex differences were noted in response to GLP-1R activation but especially in the response to GLP-1R blockade or silencing. Females showed a similar but somewhat less potent feeding and intake inhibition after LH-targeting Ex4 microinjections, results consistent with LH GLP-1R expression which was 10-fold higher in adult males compared with females, an age-dependent sex difference. This is somewhat surprising as we have previously shown that females show a more potent feeding and food motivation inhibition after intracerebroventricular Ex4 injections.[51] However, our more recent data indicate that the LH is not a neural substrate mediating this enhanced reward response.[29] In stark contrast to the effects found in males, the LH does not seem to be a critical site for GLP-1 control of motivated behavior in females, as both the acute pharmacological and chronic genetic GLP-1R inhibition in the LH did not alter food motivation in female rats. Thus female brains may more readily compensate for the reduced LH GLP-1 signal. The molecular signature of LH GLP-1R activation was also partly sex divergent, with ILs and MCH affected in both sexes, while reduced expression of orexin and neurotensin detectable only in females, selectively in their estrus phase. In



fact, the estrus phase had a surprisingly potent effect on both molecular and behavioral outcomes of LH GLP-1R activation, which were nearly completely attenuated in females in cycle phases associated with low estrogen signaling.

Our data demonstrate that, contrary to previous findings, GLP-1R in the brain, and specifically in the LH, are indispensable for the regulation of body weight and body fat in males and females. Likewise, they are essential to curb food reward behavior

Figure 5. Glucagon-like peptide-1 receptor (GLP-1R) in the lateral hypothalamus (LH) are necessary for normal body weight, body fat and food intake control in males and young females but not older females. Decreased GLP-1R expression in the LH promoted a marked, chronic, hyperphagia and weight gain in male (**a**, **d**, **g**) but not in female (**b**, **e**, **h**) rats (both sexes 13 week old on injection day). In contrast to the 13-week-old females, younger females (9 week old) presented with a massive hyperphagia (**c**, **f**) and weight gain after LH GLP-1R KD (**i**). Likewise, male knockdown rats have double the amount of fat (**j**) compared with controls, while fat mass in same age females was unaffected by the treatment (**k**). However, the adipose tissue weight of younger females with LH GLP-1R knockdown was nearly threefold higher compared with control rats (**l**). Fasted blood glucose levels are not altered by the knockdown in either sex (13 week old; **m**). Potential compensatory adaptations of the central GLP-1 system were assessed by determining whether the GLP-1 precursor (preproglucagon) expression in the nucleus of the solitary tract (NTS), or GLP-1R expression in the nucleus accumbens (NAc) or in the NTS, were altered in response to the LH GLP-1R knockdown. The expression of preproglucagon was increased in all three knockdown groups tested, thus irrespective of sex, age or weight gain response to the knockdown (**n**). A compensatory increase in GLP-1R expression was detected in NAc, a nucleus with dense connections to the LH, but interestingly this change was only detected in males (**o**). This elevation in males seemed to be area specific as GLP-1R expression in the NTS was not altered in males or any of the female groups. Thus chronic loss of LH GLP-1R, induced by an adeno-associated virus (AAV)-shRNA (short hairpin RNA) GLP-1R-mediated knockdown, not only increased food reinforcement behavior but also led to a marked hyperphagia and weight gain, at a level unparalleled to that found by blockade of any other GLP-1R population in rats or mice, despite compensatory changes detected outside of the LH. In conclusion, GLP-1R in the LH is a key component of normal body weight homeostasis and food reward control; LH emerges as one of the most critical sites for the endogenous GLP-1 effect on energy balance in males. Data are expressed as mean ± s.e.m. $n = 10$ (male rats, 5 in each treatment group); $n = 10$ (female rats, 5 in each treatment group) for 13-week-old rats and $n = 16$, 8 in each treatment group, for 9-week-old rats. GWAT: gonadal white adipose tissue, IWAT: inguinal (subcutaneous) white adipose tissue mass. M and F, males and females, respectively (13 week old at the time of AAV-shRNA infusion, and 17–18 week old at the time of tissue collection), YF: younger females, 9 week old at the time of infusion, and 15 at the time of tissue collection. Shaded gray area indicates a period of operant testing performed to capture a potential interaction of the knockdown with the estrous cycle. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

in males. Considering the robust and persistent changes obtained with this single gene manipulation in the LH, this nucleus has a tremendous potential to contribute both to the development of obesity and possibly to identifying strategies to control overeating.

CONFLICT OF INTEREST

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