The Impact of Glucagon-Like Peptide-1 on the Brain-Reward System and Beyond

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The only true wisdom is in knowing you know nothing.

Socrates

Abstract

Glucagon-like peptide-1 (GLP-1), produced in the intestine and the brain, regulates food intake and glucose metabolism. A GLP-1 based treatment is already approved for clinical use in type 2 diabetes patients, and recently in obese patients. GLP-1 fibers also form a circuitry in the brain ascending from the hindbrain, mainly nucleus of solitary tract (NTS), to the key nodes of the mesolimbic reward system (including ventral tegmental area, VTA, and the nucleus accumbens, NAc). The GLP-1 receptor is also expressed in the VTA, NAc, and amygdala.

The aim of this thesis was: 1) to investigate the impact of GLP-1 on the food/alcohol oriented behavior, 2) to identify the neuroanatomical substrate underlying the effect of GLP-1 on reward behavior.

Our results demonstrated that GLP-1 and its clinically approved analogue, exendin 4, suppressed the rewarding value of food and alcohol. GLP-1R activation in the VTA was sufficient for this effect. Amygdala has long been implicated in emotional processing, learning and memory but less attention has been given to its role in feeding behavior. Dopamine is a neurotransmitter implicated in reward and motivated behavior. Our results revealed that GLP-1R stimulation elevated the level of dopamine metabolites and dopamine turnover in amygdala. These changes were also associated with a reduction in sucrose-driven food-reward behavior.

A potential link between GLP-1 and cytokine signaling outside of the CNS has recently been suggested by a study showing an increase in GLP-1 production and secretion in response to elevated interleukin-6 in the blood. However, the relationship between GLP-1 and central cytokines remained unexplored. To determine if there is an interaction between GLP-1 and interleukin-6 (IL-6) and interleukin-1β (IL-1β) at the level of the CNS, we used both pharmacologic and non-pharmacologic (transgenic mice) models of signaling blockade for both of these cytokines. Our results indicated that IL-6 and IL-1β are mediators of GLP-1 anti-obesity effect.

Collectively this thesis revealed a potent impact of GLP-1 and its stable analogue on food and alcohol reward behavior and identified the neurocircuitry and neurochemical mediators involved. Furthermore a surprising link, relevant for the anorexic and weight loss effects of GLP-1, between GLP-1 and cytokines was discovered. Considering that GLP-1 analogues are approved for clinical use, these findings may be of considerable clinical significance.

List of papers

The thesis is based on the following 4 papers:

Paper I. **The glucagon-like peptide 1 (GLP-1) analogue, exendin-4, decreases the rewarding value of food: a new role for mesolimbic GLP-1 receptors.**
Dickson SL, Shirazi RH, Hansson C, Bergquist F, Nissbrandt H, Skibicka KP.
Journal of Neuroscience. 2012 Apr.

Paper II. **Dopamine signaling in the amygdala, increased by food ingestion and GLP-1, regulates feeding behavior.**
Anderberg RH, Anefors C, Bergquist F, Nissbrandt H, Skibicka KP.
Physiology of Behavior. 2014 Sep. Epub 2014 Feb 21.

Paper III. **Gut peptide GLP-1 and its analogue, Exendin-4, decrease alcohol intake and reward.**
Shirazi RH, Dickson SL, Skibicka KP.
PLoS One. 2013 Apr.

Paper IV. **Glucagon-like peptide 1 receptor induced suppression of food intake, and body weight is mediated by central IL-1 and IL-6.**
Shirazi R, Palsdottir V, Collander J, Anesten F, Vogel H, Langlet F, Jaschke A, Schürmann A, Prévot V, Shao R, Jansson JO, Skibicka KP.
Proc Natl Acad Sci U S A. 2013 Oct.

Papers not included in this thesis

1. **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.

2. **GLP-1 receptor stimulation of the lateral parabrachial nucleus reduces food intake: neuroanatomical, electrophysiological, and behavioral evidence.**
   Richard JE, Farkas I, Anesten F, Anderberg RH, Dickson SL, Gribble FM, Reimann F, Jansson JO, Liposits Z, Skibicka KP. Endocrinology. 2014 Nov.

3. **Divergent circuitry underlying food reward and intake effects of ghrelin: dopaminergic VTA-accumbens projection mediates ghrelin's effect on food reward but not food intake.**
   Skibicka KP, Shirazi RH, Rabasa-Papio C, Alvarez-Crespo M, Neuber C, Vogel H, Dickson SL. Neuropharmacology. 2013 Oct.

4. **Ghrelin influences novelty seeking behavior in rodents and men.**
   Hansson C, Shirazi RH, Näslund J, Vogel H, Neuber C, Holm G, Anckarsäter H, Dickson SL, Eriksson E, Skibicka KP. PLoS One. 2012.

5. **Ghrelin interacts with neuropeptide Y Y1 and opioid receptors to increase food reward.**
   Skibicka KP, Shirazi RH, Hansson C, Dickson SL. Endocrinology. 2012 Mar.

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Abbreviations

6-OHDA	6-hydroxydopamine
α-MSH	α-melanocyte stimulating hormone
AF 12198	IL-1R antagonist
AP	Area postrema
ARC	Arcuate nucleus
BLA	Basolateral amygdala
CART	Cocaine- and amphetamine-regulated
CeA	Central nucleus of amygdala
CKK	Cholecystokinin
CNS	Central nervous system
CPP	Condition place preference
DAMGO	D-Ala2,Nme-Phe4,Glyol5-enkephalin
DDP- IV	Ddipeptidyl peptidase IV
DVC	Dorsal vagal complex
DVN	Dorsal motor nucleus of vagus
ETC	Eticlopride hydrochloride
EX4	Exendin-4
EX9	Exendin-3(9-39)
FR	Fixed ratio
GLP-1	Glucagon-like peptide-1
GLP-1R	GLP-1 receptor
GLUTag	Pro-glucagon expressing cell line
HFD	High-fat diet
HPLC	High performance liquid chromatography
ICV	Intracerebroventricular
IL (IL-1, IL-6)	Interleukin
IL-1R	Interleukin-1 receptor
IL-6ab	IL-6 antibody

IL-6R	Iinterleukin-6 receptor
I.P.	Intraperitoneal
LH	Lateral hypothalamus
LPS	Lipopolysaccharide
LV	Lateral ventricle
mPFC	Medial prefrontal cortex
NAc	Nucleus accumbens
NMDA	N-methyl-D-aspartate
NPY	Neuropeptide Y
NTS	Nucleus of solitary tract
PBN	Parabrachial nucleus
PC	Prohormone convertase
PFC	Prefrontal cortex
POMC	Pro-opiomelanocortin
PR	Progressive ratio
PVN	Paraventricular nucleus
PYY	Peptide YY
QNP	Quinpirole hydrochloride
SN	Substantia nigra
SOCS 1	suppressor of cytokine signaling 1
STAT 3	Transducer and activator of transcription 3
T2DM	Type 2 diabetes mellitus
TH	Tyrosine hydroxylase
TNF-α	Tumor necrosis factor-α
VMH	Ventromedial hypothalamus
VMN	Ventromedial nucleus
VTA	Ventral tegmental area

Introduction

Obesity

Overeating is one of the key components that leads to obesity and results in imbalance between energy intake and expenditure. Due to increasing worldwide prevalence of obesity and its co-morbidity with other diseases such as type 2 diabetes mellitus (T2DM) and cardiovascular diseases, research to find an effective pharmacotherapy to treat obesity and overeating is desperately needed. Despite the fact that a wealth of literature on this topic has increased our understanding regarding regulation of energy balance and food intake control, there still is no effective anti-obesity treatment available.

So far the current most effective obesity treatment is bariatric surgery; currently available non-surgical, pharmacological options are far less effective. The past non-surgical treatments resulted in body weight loss but they also affected mood and in some cases induced anxiety and depression (1). Bariatric surgery, such as gastric by-pass, is the most effective treatment resulting in sustained weight loss by inducing appetite reduction and increase in energy expenditure (2), however as with all surgical procedures there are significant risks of performing surgery, thus ideally the surgery should be replaced with an equally effective combination of pharmacotherapeutics. Following gastric by-pass, changes in several gut hormone levels have been found, they include a decrease in circulating ghrelin and an increase in glucagon like-peptide- 1 (GLP-1) (3).

Feeding behavior

Food intake and energy expenditure are complex processes thought to be regulated by two parallel pathways, ***homeostatic*** and non-homeostatic, which is also referred as ***hedonic*** (4). Homeostasis means to maintain an energy balance in the body, where generally energy in equals energy out, but hedonic mechanisms can override this as the food becomes more palatable and rewarding.

Within the central nervous system (CNS), several areas have been identified to control feeding behavior, the two key areas are the hypothalamus and the brainstem.

Hypothalamus

Over decades studies have shown that the hypothalamus and its peptides play a key role in regulating food intake and energy balance (5, 6). The hypothalamus is divided into several subnuclei that include: lateral hypothalamus (LH), ventromedial hypothalamus (VMH), arcuate nucleus (ARC), paraventricular nucleus (PVN), and ventromedial nucleus (VMN). Between these nuclei, there is a complex neuronal network that results in orexigenic and anorexigenic effects. While lesion in VMN will induce hyperphagia (7), lesion in LH will induce hypophagia (8, 9). Based on these results, researchers call LH the ***hunger center*** and VMN the ***satiety center.***

Brainstem

For a long time, it was believed that the hypothalamus is the only region involved in the regulation of food intake. However evidence from the work of Grill and colleagues has shown that the caudal brainstem, namely the dorsal vagal complex (DVC), is also involved in ingestion and food intake regulation (10, 11). There is a reciprocal neural connection between the hypothalamus and the brainstem, and many peripherally produced circulating factors can reach both the hypothalamus and the caudal brainstem via for example the leaky blood brain barrier found in the circumventricular organs (12, 13).

The DVC consists of the nucleus of solitary tract (NTS), area postrema (AP), and the dorsal motor nucleus of vagus (DVN). The NTS receives visceral afferents from the gastrointestinal tract. Visceral afferents in the form of the vagus nerve terminate in the NTS (14, 15). Activating the vagal afferents via gastric distension, injection of nutrients in the intestine or free feeding induces c-fos in the NTS (16-19).

The gut-brain axis

The CNS is implicated in control of long-term energy balance. To do this, the brain has to be able to monitor both the level of energy intake and expenditure. There are different hypothesis about how the brain is set up to achieve this; in 1967, Mayer & Thomas proposed the *glucostatisc hypothesis* which suggested that small changes in plasma glucose levels will initiate or terminate the feeding. However this model did not provide information about which mechanism in the CNS that determines the amount of food to be consume, and it also correlated

poorly with energy expenditure (20). The second hypothesis, the *lipostatic model* was suggested by Kennedy in 1953, and hypothesized that signals secreted in proportion to the body fat mass will modulate amount of food eaten (21).

The results from the parabiosis studies on rats with a lesioned hypothalamus support the existence of humoral signals in animal's lipostatic state (22). The parabiosis involves joining two live animals by surgery in a way that allows for exchange of the humoral signals via blood. In one version of this experiment one of these animals had a lesion in the VMH. The rats with lesions started to develop obesity while those without a lesion became hypophagic and lost weight. These findings support the lipostatic model stating that the signals in proportion to the amount of fat mass inhibit food intake. There is a body of literature that supports the lipostatic hypothesis.

The brain reward system

The brain reward circuitry or cortico-limbic system is a complex system that contains the ventral tegmental area (VTA), the nucleus accumbens (NAc), the hippocampus, the ventral striatum, the prefrontal cortex (PFC), and the amygdala (23). Injection of drugs of abuse (nicotine, alcohol, and opioid) into the meso-cortico limbic system increases dopamine levels in this system (24).

Hedonic feeding focuses on the rewarding value of food. When a sated individual overconsumes food due to its palatability (high-fat or high-sugar food for example) this can lead to obesity. Since the discovery of the brain reward system (25), it has been of great interest to map out those nuclei that are involved in motivation and reward behavior. Electrical stimulation or intracranial infusion of drugs into the reward related structures will affect the same area as drugs of abuse (26). A wealth of evidence suggests that a similar response in the brain reward pathway seen after taking drugs of abuse is responsible for the hedonic aspects of food intake (27-29).

Ventral tegmental area, VTA

The VTA is a key nucleus in the mesolimbic system and plays a major role in reward seeking behavior (30). Intracranial self-administration of morphine and amphetamine into the VTA suggests that the VTA is the key node in reward

behavior controlling neurocircuitry (31). The VTA contains the cell bodies of dopamine neurons, which project to nuclei such as NAc, PFC, and amygdala.

In 1980, Wise and colleagues suggested that drugs of abuse act on dopaminergic neurons in the brain reward circuitry (32). Dopamine, a catecholaminergic neurotransmitter, affects many physiological functions such as movement, emotion, and reward behavior. Dopamine release can also be triggered by natural and/or chemical cues like feeding, sex or addictive drugs. Animal studies have shed some light on dopamine's role in feeding behavior. Thus, animals lacking dopamine production, using neurotoxin 6-hydroxydopamine (6-OHDA), show hypophagia and body weight loss (33).

Tyrosine hydroxylase (TH) is a rate limiting enzyme in dopamine biosynthesis (34) and has been used as a marker to localize dopamine neurons within the mesolimbic system. Food intake is significantly reduced in mice lacking TH but injection of L-dihydroxyphenylalanine (L-DOPA, a precursor in dopamine synthesis) will induce feeding again (35). Another study in mice lacking TH demonstrates that these mice display a preference for sucrose solutions compared to water but the total amount of consumed sucrose is still less than in the control group (36).

There is extensive evidence that palatable food (such as sucrose pellets or solutions) will increase the firing of dopamine neurons in the VTA which results in increase of dopamine levels in the NAc (37). Experiments in rodents suggest that orexigenic signals (for example ghrelin) will activate VTA dopamine neurons and release dopamine in the NAc when the rodents are exposed to food whereas this dopamine activation is inhibited by anorexigenic signals such as leptin (30, 38). In addition, NTS GLP-1 neurons project to VTA and GLP-1 receptors (GLP-1R) stimulation in the VTA can decrease food intake (39).

Since the discovery that food can engage the VTA dopaminergic neurons, many studies have followed up on this connecting with respect to food. But not all studies indicate a simple relationship of orexigenic factors increasing dopamine levels and anorexigenic factors decreasing it. For example, Lindblom and colleagues have shown that chronic food restriction will increase the mRNA level of TH in the VTA (40). More recent studies demonstrate that GLP-1R stimulation will increase the level of TH in the VTA (41, 42). Chronic high-fat feeding decreases the mRNA level of TH in the VTA (43) while chronic alcohol intake increases this level (44). Thus, the relationship of dopamine and food is quite complex and requires further investigation.

The VTA also contains γ-aminobutyric acid (GABA) neurons that project to PFC and NAc (45, 46). The VTA also receives GABAergic projections from NAc (47). A growing body of data suggests that VTA GABA neurons have an effect on VTA dopamine neuron activity. Microinjection of GABA into the VTA decreases dopamine firing whereas VTA injection of GABA antagonist increases VTA dopamine activity and dopamine levels in the NAc (48). By using the novel optogenetic methodology, selective activation of VTA GABA neuron reduces the activity of VTA dopamine neurons and dopamine release in the NAc (49, 50).

A small population of glutamate neurons is found within the VTA (51, 52) and these neurons project to the NAc, PFC, amygdala, ventral pallidum, and lateral habenula (53, 54). The VTA expresses N-methyl-D-aspartate (NMDA) receptor and glutamate exerts its effect partly through this receptor which in turn activates the dopamine neurons (55).

Pharmacological studies can help us understand the mechanism of mesolimbic dopamine and the new developed technique, optogenetics, has made it possible to further understand brain function in awake behaving animals. Non-contingent, high-frequency optical activation of VTA dopamine neurons induces condition place preference (CPP) (56). Further studies showed that non-contingent VTA dopamine activation enhances positive reinforcement in operant responding for food but has no effect in the absence of food (57).

The LH is another brain region important in reward function that interacts with the VTA and the NAc. The VTA sends dopaminergic projections to the LH and in return receives orexin projections from the LH (58). Orexin neurons, found only in the LH, play a crucial role in reward seeking behavior; in the VTA, they activate the mesolimbic dopaminergic neurons (59). The key role of orexins in drug, alcohol and food-reward is well established (60-62). For example, c-Fos activation in the VTA by high-fat diet (HFD) is abolished by peripheral blockade of orexin signaling (63). Stimulation of the LH region also leads to an increased level of both dopamine and glutamate in the VTA (64). The NAc μ-opioid receptor activation stimulates the HFD consumption by activation of the LH orexin neurons; this effect is attenuated by blocking the VTA orexin signaling (62). These findings further support the interaction of this hypothalamus with the mesolimbic reward system.

Nucleus Accumbens, NAc

The NAc is a key node in the mesolimbic system and plays a crucial role in both natural (food, sex) and chemical (such as cocaine, alcohol) reward and motivated behavior. The NAc receives glutamatergic inputs from brain regions such as the amygdala, hypothalamus, hippocampus, and PFC (65). In turn, the NAc projects to the basal ganglia, an area involved in motor behavior (65). In addition to the glutamatergic inputs, the NAc is also innervated by the VTA dopamine neurons and activation of the neurons increases dopamine release in the NAc (37).

The NAc is a heterogeneous nucleus and consists of two substructures, shell and core (66) NAc core is involved in learning and reward-associated conditioning, while the NAc shell is a link between the mesolimbic circuitry and the hypothalamus and is involved in unconditioned behavior such as food intake and hedonic response to unconditioned stimuli such as sucrose (67, 68). Stimulation of NAc shell decreases food consumption (69).

It is well established that manipulation of NAc will affect food intake (70). Ingestion of food and food reward associated cues increase dopamine levels in the NAc (71). The injection of 6-OHDA or dopamine antagonist into the NAc core but not the shell suppresses lever pressing for preferred food. In contrast, it increases the intake of normal chow (72, 73).

D-Ala2,Nme-Phe4,Glyol5-enkephalin (DAMGO), a μ-opioid receptor agonist, increases intake of HFD when injected into the NAc (74). Intra-NAc microinjection of D-amphetamine and dopamine agonist will induce CPP (75). The dopamine depletion by 6-OHDA in the NAc attenuates the rewarding effect of self-administrated amphetamine and cocaine in operant behavior and CPP (76). Microdialysis studies show an increase in dopamine release in the NAc core but not in the shell when palatable food is presented (77). Oral sucrose intake stimulates dopamine release in the NAc measured with microdialysis (78).

Optogenetic studies in mice suggest that the NAc D1 and D2 activation is not sufficient to induce CPP in the absence of cocaine (79). However, selective NAc D2 activation is sufficient to suppress cocaine-induced CPP (79).

Amygdala

The amygdala is a heterogeneous brain nucleus with several distinct neural compartments and diverse afferent and efferent projections. The amygdala receives reciprocal inputs from other brain regions such as VTA, NAc, hypothalamus and hindbrain (80). Extensive literature implicated the amygdala as a key node for emotional processing, learning, and memory (81, 82). However, the amygdala´s neuroanatomical position, neurochemical profile, and its reciprocal connection to hypothalamus, VTA, NAc, and NTS may suggest a potential role in regulation of food intake. Moreover, the amygdala expresses receptors for gut hormones such as ghrelin and GLP-1 (83, 84).

The central nucleus of amygdala (CeA) and the basolateral amygdala (BLA) are subnuclei within the amygdala. The CeA is a major target for dopaminergic projections from the VTA (85, 86) and in turn sends glutamatergic projections back to the VTA and substantia nigra (SN) (87). The BLA sends glutamatergic projections to the NAc and the medial prefrontal cortex (mPFC) (88, 89).

Bilateral amygdala lesions in female rats induce hyperphagia and body weight gain (90). Since the CeA has extensive connections with visceral nuclei in the hindbrain, it has been implicated that it might be involved in food intake regulation. In vivo studies have suggested that the CeA is involved in coding for positive reinforcement (91) while the BLA will interfere with Pavlovian conditioning , reward devaluation, CPP (92-95).

Inactivation of CeA with lidocaine in rats results in satiety resistance and impairs devaluation of pre-exposure to palatable food since the amount of eaten food is not reduced during the second meal unlike in the control group (96). However the same treatment in the BLA shows no effect on food intake (95, 97).

The neural interaction between the amygdala (CeA and BLA) and mesocortico-limbic dopamine system has been implicated in food reward behavior, and it may take place at the level of the NAc (98, 99). This idea is supported by studies demonstrating that direct injection of D2 receptor antagonist into the amygdala increases and amphetamine injection decreases dopamine levels in the NAc (100). Further amygdala dopamine lesion increases locomotor activity in response to amphetamine (101). Intra-NAc injection of opioid also increases palatable food intake. However, this effect is blocked by inactivation of the amygdala (102).

Both D1 and D2 receptors are expressed in the amygdala (103). Injection of D2 receptor antagonist stimulates food intake by increasing meal size and meal duration while D1 receptor antagonist has no effect on meals (104). By using microdialysis, peripheral injection of glucose increases dopamine turnover in the CeA (105).

One study in mice has shown that leptin, an anorexic fat-produced hormone, activates VTA dopamine projection to the amygdala, more specifically to the CeA but interestingly not to the NAc (106).

Feeding neuropeptide and gut hormones and food/alcohol reward

The body has a powerful physiologic system to regulate food intake, this system consists of both hormonal and neural pathways. Neuropeptides such as neuropeptide Y (NPY), pro-opiomelanocortin (POMC) as well as gut hormones such as GLP-1 and ghrelin are important parts of the food intake regulatory system.

The NPY, an orexigenic neuropeptide, and its receptors are found throughout the brain, mostly in the hypothalamic ARC (107). Most studies have focused on hypothalamic NPY receptor activation which increases food intake (108-110). NPY increases lever pressing (operant conditioning) for food when injected both centrally and into the LH (111, 112). Intra-NAc injection of NPY will induce CPP suggesting that NPY itself is rewarding (113).

POMC neurons are expressed in both hypothalamic ARC and in the NTS (114). Chronic but not acute stimulation of ARC POMC neurons suppresses feeding whereas acute NTS POMC stimulation rapidly decreases food intake (115). POMC depletion in the ARC but not in the NTS induces obesity and hyperphagia. Furthermore fasting reduces hypothalamic POMC mRNA expression but exogenous administration of leptin or refeeding elevates its level back to those seen in fed animals (116, 117). Mutation in the POMC gene can result in obesity and this phenotype has been observed in both humans and rodents (see ref. 118). POMC is cleaved into the α-melanocyte stimulating hormone (α-MSH), an endogenous ligand for melanocortin receptors (MCR), activation of which decreases food intake and increases energy expenditure (119, 120). Hindbrain and hypothalamic administration of MC4R agonists reduces

food intake and increases energy expenditure whereas antagonists have the opposite effect (121, 122).

So far more than 20 different gut hormones are known to be involved in the regulation of food intake. These hormones are produced either before or after each meal and can affect food intake via impact on the brain, either directly through blood circulation or indirectly via vagal afferents. These gut hormones are for example cholecystokinin (CCK), peptide YY (PYY), GLP-1, and ghrelin. CKK, PYY, and GLP-1 reduce food intake while ghrelin increases food intake. Our main focus is on GLP-1.

Glucagon-like peptide- 1, GLP-1

GLP-1, a gut peptide, is expressed in intestinal L-cells, pancreatic α-cells and in the NTS of the brain (123). Pro-glucagon is a precursors for GLP-1 and a few other gut peptides and is cleaved by prohormone convertase (PC) (124). Posttranslational processing of pro-glucagon is different in different tissues. In the pancreatic islet cells, pro-glucagon is cleaved by PC2 and yields glucagon while in the intestine PC 1/3 produces four different peptides; GLP-1, GLP-2, glicentin, and oxyntomodulin (124).

GLP-1 has many different physiological functions (125). GLP-1 is involved in regulation of gastrointestinal function by reducing gastric emptying, motility, and secretion (126). One of the main functions of GLP-1 is its insulinotropic action. Thus, GLP-1 increases insulin production and secretion from pancreatic islet cells and decreases glucagon secretion (127).

Plasma level of GLP-1

GLP-1 is released in response to nutrients and its plasma levels are low in fasting state (125). Plasma concentration of GLP-1 is significantly higher after a liquid meal than a solid meal of identical composition (128). Studies in pro-glucagon expressing cell line (GLUTag) indicate that GLP-1 secretion is stimulated directly by glucose. In vivo, fat and proteins can also stimulate GLP-1 secretion (129-131). However, proteins or amino acids alone fail to induce GLP-1 release (123). These results are not consistent since a study in GLUTag suggests that glutamine, a protein metabolite, may potently increase plasma GLP-1 (132). Lipase is an enzyme which hydrolyses lipids to yield free fatty

acids and triglyceride. Inhibition of lipase suppresses GLP-1 release (133). Furthermore, oral but not intravenous infusion of lipids elevates the level of plasma GLP-1 (134).

In the periphery, GLP-1 is rapidly degraded and inactivated by the enzyme dipeptidyl peptidase IV (DDP- IV), a process which generates GLP-1 (9-36) amid or GLP-1 (9-37) amid (135). These metabolites are inactive but they can act as a competitive antagonist at GLP-1R (135, 136). Thus this rapid elimination by DDP-IV can be responsible for the short half-life of plasma GLP-1 which is about 1-2 minutes (137).

GLP-1 Receptor, GLP-1R

GLP-1R is a G protein-coupled receptor and is widely distributed in both the periphery and the brain (125). GLP-1R was first cloned from rat pancreatic islet cells and later from human islets (138, 139). Later studies demonstrate that the lizard peptide, exendin 4 (EX4), is a potent agonist of the GLP-1R and the truncated peptide exendin (9-39) (EX9) is an antagonist of GLP-1R (139, 140). In the periphery, GLP-1R is located in the portal vein, liver, pancreatic β-cells, and vagal afferent (141, 142).

Since the discovery of GLP-1 producing neurons in the brain, more specifically in the NTS (143), GLP-1 fiber projections were found to different nuclei within the brain with the highest concentration in the hypothalamus (144). GLP-1R are found in several brain nuclei including hypothalamic ARC, PVN, DMH, the parabrachial nucleus (PBN), AP, and VTA; all are well known to be involved in the regulation of food intake and motivated behavior (83, 144-146). Central injection of GLP-1 induces c-Fos activity in the hypothalamic PVN and the CeA. However, EX9 inhibits c-Fos activity in these areas (147).

Brain impact of GLP-1 on food intake and body weight control

To understand the physiological role of GLP-1 on food intake and body weight, studies are performed by either intracerebroventricular (ICV) or site specific microinjections in rodents. It is well established that central injection of GLP-1 or EX4 will suppress food intake (147, 148). Early studies demonstrate that ICV

injection of GLP-1 decreases food intake in chow-restricted rats (147, 149). EX9 by itself has no effect on food intake in food restricted rats but will increase the amount of food eaten in fed animals. Thus it blocks the effect of GLP-1 induced food intake suppression (147). Chronic ICV injections of GLP-1 reduce both food intake and body weight while chronic ICV injections of EX9 produce an increase in feeding and body weight (150).

Intraperitoneal (I.P.) administration of EX4 or liraglutide (another GLP-1R analogue) induces food intake suppression; this effect is blocked by central delivery of EX9 (142). Also, GLP-1 injection into the PVN attenuates food intake without inducing aversion (151). Involvement of NTS/hindbrain GLP-1 in the suppression of food intake is also demonstrated by Hayes and colleagues (152, 153). In both supracollicular decerebrate and sham rats, food intake is attenuated by exogenous GLP-1 injection into the 4th ventricle. This result indicates that activation of hindbrain GLP-1R is sufficient to induce food intake suppression (153). However, the 4th ventricle injection of EX9 increases food intake after refeeding fasted animals (152). These studies establish GLP-1 effect on the homeostatic regulation of food intake. However GLP-1 role in food reward and food motivated behavior remains to be investigated.

GLP-1 and food reward

Anterograde tracing reveals that ascending GLP-1 fibers from caudal NTS terminate in the VTA and the NAc (154). In addition, GLP-1R mRNA is expressed in these nuclei (83, 145). Thus, the role of GLP-1 on food reward and food motivated behavior is a topic of great interest.

GLP-1 injection into the NAc core increases c-Fos expression in this area (155). The same result is obtained after peripheral injection of EX4, which induces c-Fos activation in the NAc and the CeA (156). The c-Fos activation in the core suggests a possible role in food intake. Only intra-NAc core not the shell injection of GLP-1 decreases food intake while EX9 injection into the NAc core induces hyperphagia (155).

Pre-proglucagon neurons in the NTS project directly to the VTA (39). Intra-VTA or NAc core injections of EX4 decrease sucrose intake in overnight fasted rats (157). When satiated rats have a choice between HFD and normal chow, intra-VTA EX4 injection reduces HFD intake but increases the intake of

normal chow intake. But when they only have access to normal chow, VTA or NAc EX4 injection has no effect on food intake (39). In addition, to the VTA EX4 induced suppression of HFD, the intra-NAc core EX9 injection stimulates sucrose intake compared with control (157). These results suggest that mesolimbic GLP-1R activation might reduce food intake by decreasing the rewarding value of highly palatable food, a hypothesis that will be pursued in this thesis.

GLP-1 and alcohol reward

Alcohol is a product of sugar fermentation. Alcohol, like other psychostimulants, is addictive but it can also provide calories. Studies have shown that gut-brain signaling may affect alcohol intake. Central injections of ghrelin, an orexigenic gut peptide, increase while ghrelin antagonist attenuates alcohol intake (158). The first link between GLP-1 and alcohol intake surfaced in a gastric bypass study showing that alcohol intake is reduced after gastric bypass in both human subjects and ethanol-preferring rats. This effect is associated with an elevated plasma level of GLP-1 in alcohol-preferring rats. Notably, I.P. administration of EX4 decreases alcohol intake in sham alcohol-preferring rats (159).

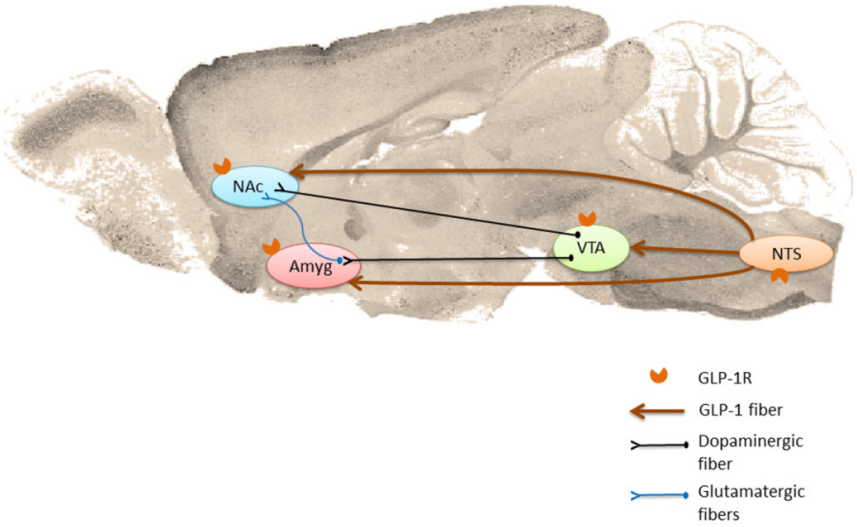


Figure 1. GLP-1 projection to mesocortico-limbic system. NTS (Nucleus of solitary tract), VTA (Ventral tegmental area), NAc (Nucleus accumbens), and Amyg (Amygdala).

GLP-1 and thermoregulation

Maintenance of body temperature in a critical range is crucial for mammalian survival. Body temperature is regulated by an interaction between neural and hormonal signaling. Considering the widespread expression of GLP-1R within the brain, it is possible that GLP-1 can affect the thermoregulation as well. GLP-1 effect on body temperature was first demonstrated by O´Shea and his colleagues in 1996 (160). They showed that ICV GLP-1 injection decreases both body temperature and food intake and these effects are blocked by prior ICV EX9 injection. However, ICV EX9 injection itself has no effect on temperature (160). The same result is seen after I.P. or 4th ventricle administration of EX4 in both control and supracollicular decerebrated rats (153). The hindbrain injection of cocaine- and amphetamine-regulated peptides (CART) induces hypothermia and pretreatment with EX9 abolishes this effect (161). Both these findings implicate GLP-1 role in thermoregulation.

Obesity and the circulating inflammatory factors

There may be a close link between metabolism and immunity. Inflammation induced by obesity is distinct from the classical inflammation described as redness, swelling, heat, and pain (162). Obesity induced inflammation is first seen in obese mice, which display a high level of tumor necrosis factor-α (TNF-α), a pro-inflammatory cytokine, in their adipose tissue compared with the lean control mice. Obese mice lacking TNF-α have improved insulin sensitivity and glucose homeostasis (163). This significant finding led to both animal and human studies exploring the inflammation state in obese and lean subjects.

It is well known that besides TNF-α, other inflammatory factors including interleukin-6 (IL-6) and interleukin-1β (IL-1β) are also increased in the adipose tissue of obese subjects (164). However the increased level of cytokines is not limited to obese adipose tissues; other organs such as brain, liver, and pancreas show an elevated cytokine expression in obese state (165). Over the last decade a growing body of evidence has implicated that both IL-6 and IL-1 may play an important role in regulation of metabolism.

Interleukin-6, IL-6

IL-6 is a multifunctional cytokine, which plays a role in both physiology and pathophysiology. Perhaps it is best known as a pro-inflammatory factor which is released from immune cells in response to inflammation (166). However, IL-6 is also produced from adipose tissue in a non-inflammatory condition in humans and the plasma level of IL-6 correlates with fat mass (167). Later studies detected an expression of IL-6 and the IL-6 receptor (IL-6R) at the level of the CNS (168, 169). Furthermore, mice lacking IL-6 develop mature onset obesity and treatment with IL-6 partially reverses this effect. Interestingly, only ICV, not I.P., injection of IL-6 increases energy expenditure (170) and chronic ICV treatment with IL-6 decreases intake of HFD and body weight (171).

Interleukin-1, IL-1

IL-1 is a major pro-inflammatory cytokines and it is released in response to infection or inflammation. IL-1 and IL-1 receptor (IL-1R) are expressed within the brain (172-174). Peripheral or central injection of IL-1 induces thermogenesis, and decreases food intake (175). ICV injection of IL-1β suppresses food intake and body weight gain (176). Just like IL-6 knockout mice, IL-1 knockout mice also develop obesity (177, 178). Consistently with this finding, mice with IL-1R antagonist deficiency, triggering IL-1 overactivity, display a lean phenotype (179).

Mice lacking both IL-6 and IL-1 exhibit an early obesity and hyperphagia compared with mice lacking only either one of them (180) suggesting a potential interaction between these two interleukins.

The link between GLP-1 and cytokines

Evidence suggests a link between GLP-1 and cytokines in the periphery. Elevation of plasma IL-6 is associated with exercise, obesity and T2DM. IL-6 release during exercise improves insulin sensitivity and increases production and secretion of GLP-1 from intestinal L-cells and pancreatic α-cells (181). Both IL-6 and IL-1β triggered GLP-1 secretion leads to an increased insulin secretion and lowered blood glucose (182). However, GLP-1 seems to inhibit the lipopolysaccharide (LPS)-induced IL-1β production in astrocyte cell culture (183). Systemic LPS injection induced food suppression is attenuated by 4th ventricle delivery of EX9, which implies that hindbrain GLP-1 signaling is a

mediator of LPS (184). Taken together these results suggest a link between cytokines and GLP-1 in both periphery and brain. They also point to a complex, potentially bidirectional interaction that may vary based on the tissue and specific brain region involved as well as the physiological context.

Clinical aspects of GLP-1

GLP-1R long lasting analogues, exenatides and liraglutide, have been approved in 2005 for treatment of patients with T2DM. They reduce blood glucose levels without inducing hypoglycemia. In contrast to other anti-T2DM pharmaceutical treatments, GLP-1R agonists significantly reduce body weight in T2DM patients (185). In fact, GLP-1R agonists can also reduce body weight in obese non-T2DM patients. Furthermore this reduction is more prominent in patients without T2DM (186, 187). Once daily treatment with liraglutide increases postprandial satiety and reduces hunger and food consumption without increasing energy expenditure (188). These findings strongly suggest the utility of GLP-1 agonists in the treatment of obesity. In fact, liraglutide has been recently approved in the USA (2014) and in Europe (2015) for weight management (185).

Aims of the thesis

The overall aim of the thesis was to investigate the role of brain GLP-1R stimulation on food oriented behavior.

Specific aims

- To investigate the central effect of GLP-1 on food motivated behavior and to determine the neurobiological substrates underpinning this effect.

- To determine the role of GLP-1 on alcohol intake and reward and discover the neuroanatomical substrates underlying these effects, focusing on the ventral tegmental area.

- To study the impact of central GLP-1R stimulation on amygdala dopamine turnover and whether these changes can contribute to the anorexic effect of central GLP-1R activation.

- To investigate a link between GLP-1 and cytokines at the level of the CNS and whether the effect of GLP-1 on food intake and body weight is mediated by IL-6 and IL-1.

Methodological considerations

Animals

In our studies we used two types of rat strains, Sprague-Dawley, purchased from Charles River (Germany), and Wistar rats supplied by Taconic (Bomholt, Denmark).
Sprague-Dawley rats were used for operant conditioning (paper I and II) and in CPP (paper I) while Wistar rats were used in the alcohol study for all tests except the CPP for alcohol (paper III). For alcohol-induced CPP NMRI mice (B&K Universal AB, Soletuna, Sweden) were used since both in house preliminary testing as well as previous literature data (189) indicated that rats do not reliably establish CPP to alcohol.

Genetically modified mice

Genetically modified mice are one of the most common non-pharmacological models used to study the relationship between a phenotype of a disease and a gene mutation.

Conditional transgenic mice are one of the most powerful tools to study gene functions. One advantage of using conditional transgenic animals rests in the ability to control the timing of gene modification. This potentially allows avoiding developmental compensation of a gene manipulation seen in traditional non-conditional knockout mice. Bacteriophage P1 Cre, a site-specific recombinase, is a well-established method to manipulate mammalian genome (190). In this transgenic mouse, two loxP sites are flanked around a functionally essential part of the gene of interest. These loxP sites are recognized by Cre and they allow Cre to delete the flanked segment. An experimenter can control when and where in the body Cre is applied. Cre can be delivered by Tat-Cre, which is a recombinant cell-permeable fusion protein. Tat-Cre is generated by fusing a protein transduction domain derived from HIV-1 TAT protein (TAT) and the Cre protein (191). Tat peptide enhances cellular uptake of Cre and eliminates the risk for insertional mutation (190).

We used two types of genetically modified mice, IL-1R1 knock-out and IL-6Rα LoxP/LoxP mice. These mice and their littermates C57BL/6J were supplied by The Jackson Laboratory (Bar Harbor, ME, USA).

IL-1R1 knock-out mice were backcrossed to C57BL/6J for five generation and their second filial was backcrossed for two generations. The development of these transgenic mice is described in detailed in (192).

The IL-6Rα LoxP/LoxP mice were bred for 5 months after their arrival to the animal facility of the University of Gothenburg. When they received the Tat-Cre injection, they were 5 months old. The exons 4-6 of these mice are flanked by two LoxP sites. For further explanation of this method see section below: Brain IL-6 knockdown.

All animals were housed in 12 hour light/dark cycle (lights on at 6am) with regular chow and water available *ad libitum* in their home cage unless otherwise stated. Experimental testing commenced at 10 am. All animal procedures were carried out with ethical permission (ethical permissions 334-04, 336-09, 199-11, 192-11, and 156-12) and in accordance with the University of Gothenburg Institutional Animal Care and Use Committee guidelines.

Drugs

EX4 and EX9, two non-human peptides, have been isolated from Heloderma suspectum venom (193). EX4 is a long lasting analogue of GLP-1 and a full agonist at the GLP-1R whereas EX9 acts as a highly selective antagonist at these receptors (139). In 1995 Goke and his colleagues showed that the pattern of EX4 binding sites is identical as that seen with GLP-1 in the CNS (145). Central administration of GLP-1 or EX4 inhibits feeding (147, 148). Peripheral administration of GLP-1 and EX4 reduces HFD intake (194). Conversely, EX9 can exhibit an orexigenic effect in low-fat fed rats but not in HFD fed rats (194).

The selected intra-VTA dose of EX4 was based on our own preliminary results. It was chosen to be subthreshold to ventricular application, thus this dose of EX4 was not effective in reducing operant responding while administrated ICV but, as we later showed, was effective when injected into the VTA.

Dopamine, a key neurotransmitter, has been widely investigated in feeding behavior. So far 5 different dopamine receptor subtypes have been identified, D1, D2, D3, D4, and D5. Pharmacological and behavioral evidence indicates that stimulation or blockade of dopamine receptors, D1 or D2, can affect

feeding behavior (195). Thus for one of our studies we chose to target these two receptors in an area not previously explored for its role in feeding behavior – the amygdala. The specific substances we chose to work with include: (-)-Quinpirole hydrochloride (QNP; D2 receptor agonist), Eticlopride hydrochloride (ETC; D2/D3 receptor antagonist), SKF 81297 (SKF; D1 receptor agonist), SCH 39166 hydrobromide (SCH; D1 receptor antagonist). All were purchased from Tocris (Bristol, UK).

IL-6 and IL-1 are two inflammatory factors that induce fever, reduce appetite, and physical activity (196, 197). IL-6 stimulates GLP-1 secretion which improves insulin secretion (181). It is also possible that GLP-1 and IL-1 interact (198). Past literature indicates that GLP-1, IL-6, and IL-1 decrease food intake and induce weight loss at the level of the CNS. This common impact on feeding and weight loss led us to hypothesize that these three substances may also be interacting at the level of the CNS. AF 12198, an IL-1R antagonist, and IL-6 antibody (IL-6ab; H-183) were used to determine if there is any interaction between GLP-1 and these cytokines in the brain. AF 12198 was purchased from Tocris (Bristol, UK) and IL-6ab was purchased from Santa Cruz (AH Diagnostics, California, USA). The use of these methods to block cytokine signaling was based on previous literature (199, 200).

Behavioral testing

Behaviors are defined as a range of changes in actions in response to various stimuli or inputs. In other words behaviors are the final output of the CNS activity and behavioral analysis can provide us with crucial information about the function of the CNS. Several rodent behavioral tests have been developed and used widely to better understand behavior related to reward and motivation.

Operant conditioning and CPP are the two most common behavioral models utilized to study the rewarding properties of drugs of abuse for example the motivation or the willingness of the subjects to obtain rewarding substances.

Operant conditioning

Operant conditioning is a well-established task to study food reward and food motivated behavior (201): the higher the motivation to obtain the rewarding substance the harder the rat is willing to work (press a lever) for it. Food-

induced operant conditioning training and testing were carried out in two-lever operant chambers designed for rats (Med-Associates, Georgia, VT, USA). Each chamber, situated in a light-and sound-attenuated cubicle, had a metal grid floor, two retractable levers, a white light bulb above each lever, and a food pellets dispenser for delivery of 45 mg sucrose pellet (Test diet, Glaxo-SmithKline) to the food tray. Each chamber was illuminated with a white light bulb. Data collection and processing were controlled by MED-PC software. All rats underwent a mild food restriction which gradually reduced their initial body weight to 90% during one week. They were trained to press a lever for a sucrose reward. Training was conducted in four stages: rats were first trained on fixed ratio 1 (FR1) schedule (single press on the active lever = delivery of one sucrose pellet), followed by FR3 and FR5 (3 and 5 presses per one 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 (PR). Presses on the inactive lever were recorded but had no programmed consequences. Under PR schedule, the cost of a reward was progressively increased over successive trials to determine the effort the rat will emit for it. The response requirement increased according to the following equation: response ratio = [5e^ (0.2 × infusion number)] – 5 through the following series: 1, 2, 4, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, 328 (202-204). Operant response testing was performed after the responses stabilized (number of pellets earned per session did not differ more than 15% for three consecutive sessions).

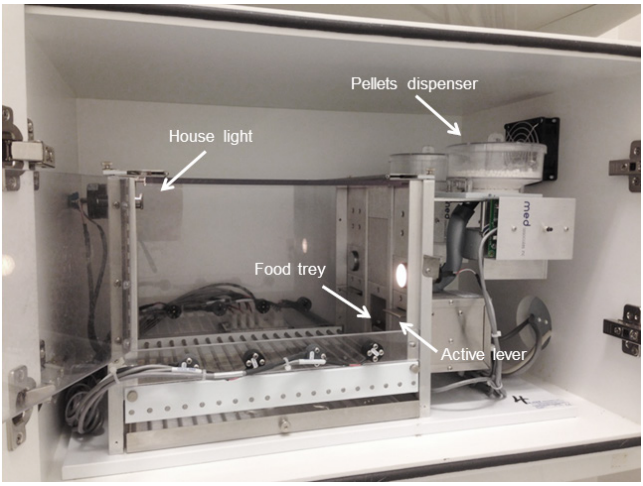


Figure 1. Schematic illustration of operant box with two retractable levers.

Conditioned place preference, CPP

The CPP test involves the preference for an environment associated with the rewarding /reinforcing effect of a treatment. The CPP apparatus consists of two connected compartments with distinct visual and tactile qualities (Med-Associates, Georgia, VT, USA). The task involves three phases; habituation, conditioning, and preference testing. During the habituation, the animals are free to explore both compartments in order to reduce the novelty of chambers and to assess an initial preference for one of the chambers. During the conditioning days, the animal has access only to one chamber and the less preferred chamber is paired with the rewarding/reinforcing substances such as chocolate or alcohol. The preferred chamber is paired with a less rewarding substance such as normal chow or saline (vehicle for alcohol). On the preference testing day, the animals were injected with either vehicle or EX4 and had free access to both compartments. The behavior of the animals was recorded by infrared beams in each chamber and time spent in each compartment was recorded. Time spent in each compartment reflects how rewarding the animal found the substance/food that was paired with that compartment.

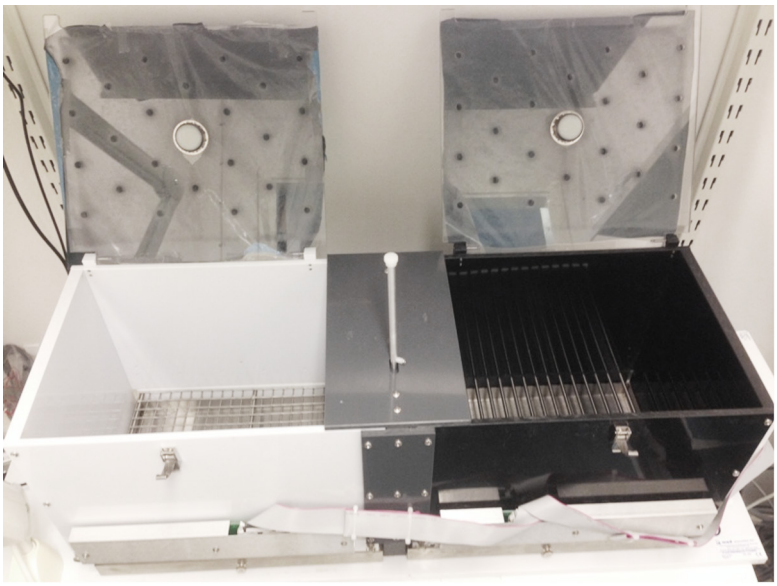


Figure 2. Standard two-chambers condition place preference box.

Food intake and body weight

GLP-1R role in controlling food intake is well established but the role of this receptor specifically in the VTA or amygdala (two sites that were specifically examined in this work) is still uncharted. Thus, in addition to reward behavior – the behavior of primary interest in many of our studies - we also measure chow intake after each reward test. In this way we can see the effect of GLP-1R activation on both food intake and food motivated behavior.

The chow was pre-measured prior to each injection and measured again post injection at 1 h and 24 h. For experiments focusing only on chow intake, food was made available 20 min after the injections and measured at 1, 2, and 24 h.

Body weight was monitored every day. All subjects were weighted right before injections and 24 h post injection.

PICA response

The most common side effects in patients with type II diabetes who undergo GLP-1 analogue treatment are nausea and illness (205). There are also preclinical studies that clearly show visceral illness and condition taste aversion (CTA) after GLP-1R stimulation in rodents (206-208). Since rodents don´t have the ability to vomit, signs of visceral illness and nausea in rats and mice can only be estimated by the Pica response or CTA. Nonetheless it is important to measure parameters related to nausea whenever feeding responses are measured in rodents in order to determine whether the reductions in feeding noted after GLP-1 stimulation are related to illness. Pica, eating of non-nutrient substances such as kaolin, is a response to nausea inducing agents in rats (209, 210). To avoid the association of kaolin to injections, kaolin was presented in the rat´s home cage for 3 days prior to the start of the experiment. An increase in kaolin intake is considered to be an illness-response behavior (210, 211).

Motor activity measurements

Besides nausea, disturbance in the locomotor activity can be another concerning effect of GLP-1 or EX4 in rodents. Some studies have shown that peripheral injection of EX4 reduces locomotor activity (212, 213) while others didn´t see any association between GLP-1 or EX4 and hypoactivity (153, 214). It is possible that this discrepancy can be explained by the dose of the GLP-1 agonist

and also the site of injection. Nonetheless, in our studies locomotor activity needs to be monitored in order to determine that the reductions in food intake are due to a physiological effect and not a general locomotor suppression. To study if there is any association between GLP-1 or EX4 induced reduced food intake or reward, spontaneous X-Y plane and rearing activity were measured during 60 min after injections and analyzed in 10 min bins (papers I and II).

Intermittent-Access 20% Ethanol 2-Bottle-Choice Drinking Model

The intermittent-access 20% ethanol-drinking paradigm has been used in alcohol addiction studies to induce high voluntary alcohol consumption in laboratory rodents. This paradigm was adapted from Wise (1973) and Simms (2008) and consists of repeated cycles of access to alcohol and withdrawals from it (215, 216). In brief, rats had access to one bottle 20% ethanol solution without sweetener and one water bottle during three 24 hour sessions per week. After each ethanol session, the ethanol bottle was replaced with a second water bottle which was available for 24 h and during the weekend. In each ethanol session, the placement of the ethanol bottle was alternated to control for side preference. Rats were weighed six days per week to calculate ethanol intake per kg of body weight.

Drug testing began 4 weeks (or total of 12 ethanol sessions) from the first ethanol exposure. This period creates a stable ethanol intake in Wistar rats, comparable with that of the alcohol-preferring rats (216). Rats had unlimited access to chow and water at all times and intake of both was measured together with the ethanol measurements. Injections were always completed 30 min before ethanol exposure.

Telemetric transponder surgery

To record body temperature and spontaneous physical activity (X-Y plane only), parameters related to energy expenditure, rats were implanted with telemetric transponders (G2 VitalView; Mini Mitter/Respironics, Bend, OR). Under ketamine anesthesia, transponders were inserted into the abdominal cavity and secured with sutures to the abdominal muscles. The use of this telemetric system allowed for continuous (every 5 min) measurements without any disturbance or stress to the animal.

Brain surgery

Over a few decades, stereotaxic surgery for implantation of cannula has become a valuable tool in neuroscience studies to investigate the effect of local manipulation of neurotransmitters, site-specific lesions or injections of drugs of interest in awake freely moving rats.

In our behavioral studies targeting the CNS, a guide cannula was positioned and attached to the skull. Under either isoflurane (Baxter, Kista, Sweden) or ketamine anesthesia, rats were placed in a stereotaxic frame to keep the skull in a fixed position. A guide cannula (26 gauge; Plastics One, Roanoke, VA), aiming at the region of interest, is implanted and fixed to the skull with dental acrylic and jeweler's screws and closed with an obturator.

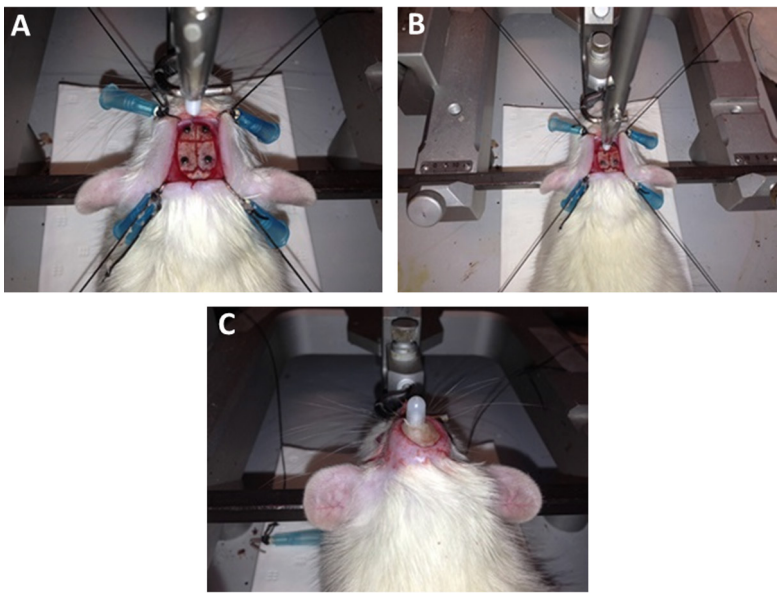


Figure 3. Brain surgery illustration. The head is fixed in the stereotax (A) and a cannula is inserted into the lateral ventricle (LV) according to the coordinates from the rat brain atlas (B). The cannula is fixed with the dental cement and closed with an obturator (C).

After surgery animals are placed into individual cages with water and food available ad libitum and allowed to recover for one week before the start of behavioral testing. In this thesis, cannulas were implanted in the VTA (paper I and III), NAc (paper I), LV (paper I-IV) and the amygdala (Paper II).

Histology

Cannula placement was confirmed post mortem by microinjection of India ink at the same microinjection volume (0.5 µl) used throughout the study. Only rats with the correct placement were included in the data analysis.

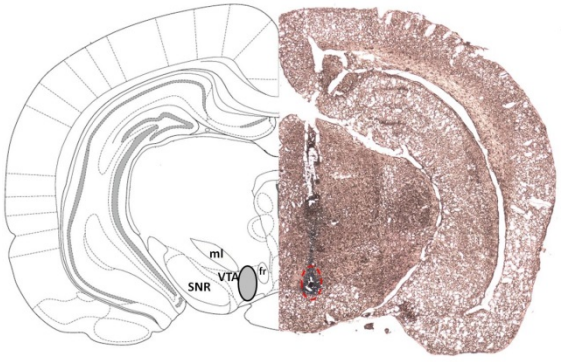


Figure 4. Histological verification of the cannula placement for the VTA microinjection. Schematic illustration of the VTA from the rat brain atlas (left) and photomicrograph of 40 µm coronal section of the rat brain (right). Abbraviations: VTA (ventral tegmental area), SNR (substantia nigra-reticular part), ml (medial lemnicus), fr (fasciculus retroflexus).

Brain IL-6R knockdown

A Tat-Cre fusion protein, synthesized as described (190), was stereotaxicaly injected into the LV (217) of 6 month old male isoflurane anesthetized mice that were homozygously floxed for IL-6Ra (Strain B6; SJL-*Il6ra*$^{tm1.1Drew}$) (218). Control mice (also B6; SJL-*Il6ra*$^{tm1.1Drew}$) were infused with vehicle (saline). Tomato expression was decreased in cells surrounding the ventricles following treatment with Tat-cre according to a similar protocol to dtTomato$^{loxP/+}$ mice.

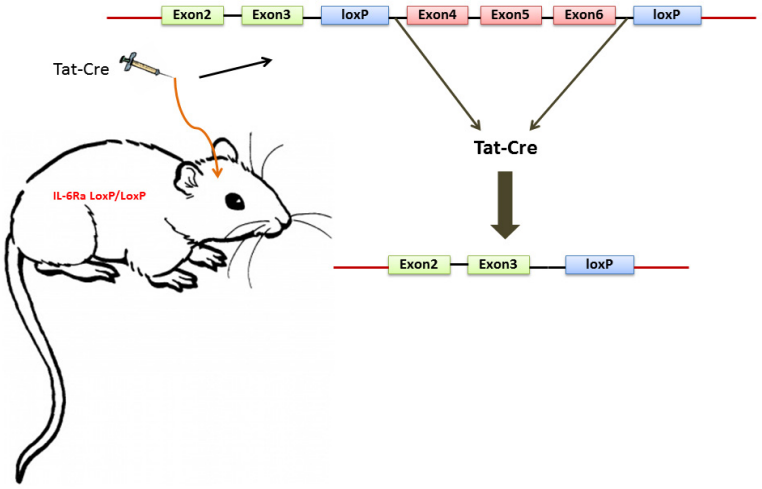


Figure 5. IL-6Ra LoxP mice. Infusion of Tat-Cre protein into the LV. Exon4-6 are flanked by loxP which undergoes site specific recombination mediated by Tat-Cre protein.

Dissection and analysis of brain tissues

Brain dissection

For gene expression study, western blot, and high performance liquid chromatography rats were lightly sedated using isoflurane and decapitated. The brains were removed rapidly and the regions of interest were dissected using a brain matrix. Dissected regions were frozen in liquid nitrogen and stored in -80° C for later gene study.

Gene expression; RNA isolation and mRNA expression

The level of mRNA of a specific gene in a specific tissue is used to measure gene expression. First RNA is purified from the specific tissue. The RNA must be transformed into complementary DNA (cDNA) by reverse transcriptase since DNA polymerase cannot use RNA as a template.

A standard protocol for gene expression, described here briefly, was used in our studies. To extract RNA, 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.

To quantify gene expression TaqMan PCR was used. TaqMan gene expression assay uses a pair of unlabeled PCR primers with a probe with a fluorescent dye label on the 5´ end, and a non-fluorescent quencher at the 3´ end. When both dye and quencher are in close contact, the dye cannot fluorescate. The denaturated cDNA binds to both the primers and the probes. As the TaqMan polymeras cleaves the probe bound to the cDNA, the fluorescent dye is released from the quencher. Hence by measuring the fluorescence, the amplification of a specific gene can be detected. Gene expression values were calculated based on the $\Delta\Delta C_t$ method (219), where the vehicle-injected group was designated as the calibrator. Beta actin was used as reference gene.

Western blot analysis

Western blot is a useful technique in research to detect, separate, and identify specific proteins from a mixture of proteins extracted from cells or tissues by using a specific antibody. The proteins in the mixture are separated by using gel electrophoresis based on their size and types. Then to detect the protein of interest, antibody detection is used.

Western blot analysis was employed to assess the activation of transducer and activator of transcription-3 (STAT 3) as indicated by phosphorylation in the hypothalamus and the hindbrain (paper IV). It was also used to detect the protein levels of endogenous suppressor of cytokine signaling (SOCS) protein, SOCS 1 and SOCS 2, in these tissues. STAT 3 is a transcription factor, encoded by *STAT 3* gene. This protein is activated in response to various cytokines such as IL-6 and can mediate the expression of SOCS which in turn can regulate cytokine signaling in the CNS (220, 221).

High performance liquid chromatography, HPLC, for measurement of amygdala dopamine

HPLC is a powerful technique in analytic chemistry used to separate the components in a mixture based on their chemical properties and to quantify

each component. To identify and quantify a component, a pressurized liquid and a sample mixture pass through a column filled with an adsorbent. The sorbent is a granular material made of solid particles, it will interact with the component in the sample mixture, which will be separated due to different interaction with the sorbent particles. The mobile phase or pressurized liquid is a mixture of solvents such as water, citric acid and or methanol.

We used HPLC to measure amygdala dopamine turnover in two separate studies (Paper II); first to assess the impact of feeding on amygdala dopamine signaling, chow was available to food restricted rats 30 min before sacrificing. In the second study, to determine the effect of central GLP-1R stimulation on amygdala dopamine turnover, restricted rats were sacrificed 30 min after injection of EX4 or vehicle into the LV.

Results and Discussion

Paper I

In this paper we demonstrated that peripheral injection of EX4 decreased food reward. Food motivated behavior was reduced after I.P. injection of EX4, as demonstrated by reduced PR operant responding for sucrose (Fig 1.1A). Furthermore, GLP-1R stimulation with EX4 abolished chocolate induced CPP (Fig. 1.1B). The mediation of the reward suppression by central GLP-1R was suggested by results indicating that ICV injection of EX4 induced a dose-dependent suppression of sucrose reward in the PR operant conditioning paradigm (Fig. 1.2).

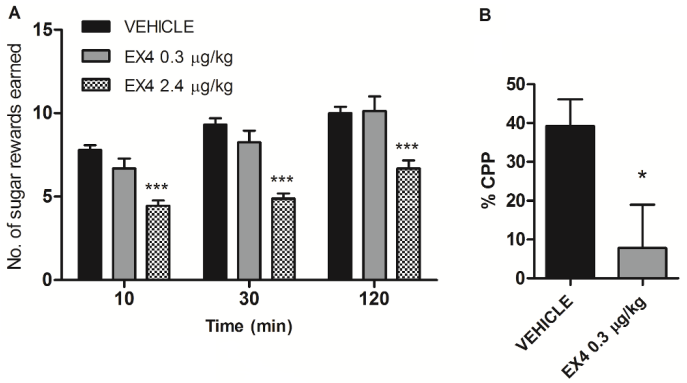


Figure 1.1. Peripheral GLP-1R stimulation decreased food reward and food motivated behavior. A) EX4 reduced lever pressing in operant conditioning compare with vehicle. N=16-48, ***p<0.0005. B) EX4 treatment attenuated preference for chamber paired with chocolate. N=9, *p<0.05. Data represent mean ± SEM.

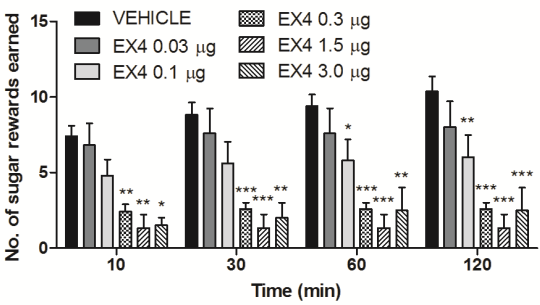


Figure 1.2. Central (ICV) injection of EX4 reduced operant responding for sucrose compared to vehicle. N=2-10. *p<0.05, **p<0.005, ***p<0.0005. Data represent mean ± SEM.

Central action of EX4 to reduce food reward behavior was confirmed by pretreatment with the GLP-1 antagonist, EX9 into the brain ventricle. To make a reliable interpretation of interaction between EX4 and EX9, we chose an EX9 dose that did not show any effect when administrated alone. Pretreatment with EX9 abolished EX4 induced reduction in sucrose reward (Fig. 1.3). This result confirms that central EX4 decreases food-motivated behavior via GLP-1R stimulation.

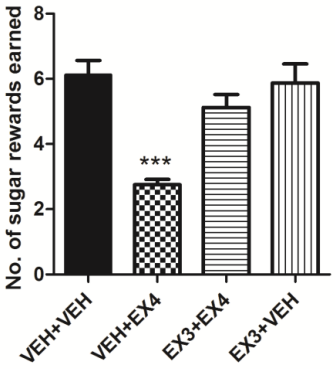


Figure 1.3. Pretreatment with GLP-1R antagonist, EX3, attenuated EX4 induced suppressive effect on lever pressing in operant conditioning. N=8. ***p < 0.0005. Data represent mean ± SEM**.**

I.P. and ICV administration of EX4 allow for a broad distribution of the drug throughout the body, and the drug may potentially diffuse into many central nuclei, so the specific site of action remains undetermined with this route of administration. This is especially true for GLP-1 since its receptors are widely distributed throughout the CNS. GLP-1R found in the NTS and the hypothalamic nuclei (e.g. LH) have previously been shown to be relevant for the anorexic effect of GLP-1 (152, 222). However, while these nuclei have indirect neuronal connection with the reward system they are not considered to be a part of the classic reward system suggested to be key in the regulation of all reward behaviors. Importantly, the NTS GLP-1 neurons project directly to the VTA and the NAc (39, 83), two key nuclei of the classic reward system. Based on these previous neuroanatomical findings, we microinjected EX4 into the VTA or the NAc to determine if these two mesolimbic nodes are direct targets for EX4 to exert its effect on food reward. Since the nature of the VTA GLP-1R stimulation on food intake is unknown, we also measured food intake 1h after

operant testing. Our results indicated that microinjection of EX4 (0.03 and 0.1 μg in 0.5 μl) into the VTA suppressed food motivated behavior at all doses and at all-time points in food-restricted rats (Fig. 1.4A). However, only the higher dose of EX4 reduced food intake (Fig. 1.4B).

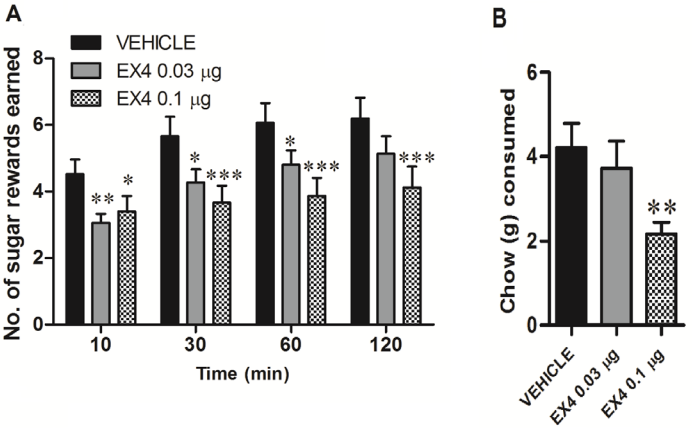


Figure 1.4. VTA GLP-1R stimulation decreases reward behavior and food intake. A) Microinjection of EX4 into the VTA reduced significantly the number of sucrose earned. B) Chow intake was decreased by the VTA EX4 microinjection. N=15-16. *p<0.05, **p<0.005, ***p<0.0005. Data represent mean ± SEM.

Direct NAc GLP-1R stimulation reduced lever pressing in an operant paradigm at all time points measured (Fig. 1.5A). In contrast to the results obtained from the VTA injections, only the higher dose of EX4 injected into NAc was sufficient to suppress operant responding for sucrose. This result implicated that the NAc might be less sensitive to EX4 compared to the VTA. Chow intake was also reduced in intra-NAc EX4 treated rats (Fig. 1.5B).

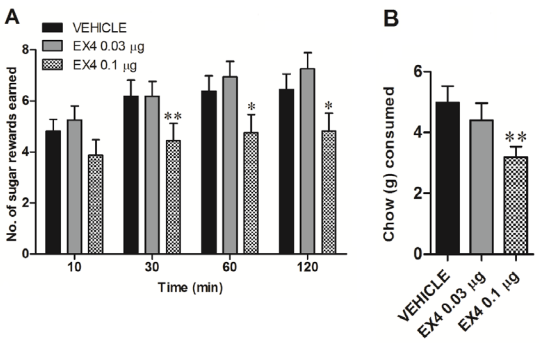


Figure 1.5. Direct NAc GLP-1R stimulation reduces food intake and food motivated behavior. A) Number of sucrose earned is reduced by intra-NAc microinjection of EX4. B) Microinjection of EX4 into the NAc suppress food intake. N = 15-16. *p<0.05, **p<0.005, ***p<0.0005. Data represent mean ± SEM.

These results are in line with a previous study showing a reduction in HFD after a direct microinjection of EX4 into the VTA and the NAc shell (39). Both our data and the previous data from (39) indicate that stimulation of GLP-1R in the VTA is more effective in reducing feeding behavior than stimulation of GLP-1R in NAc shell. The physiological role of the mesolimbic GLP-1 in the regulation of feeding behavior is demonstrated by direct injection of GLP-1R antagonist, EX9, into the VTA (39) and the NAc core (155). Both sets of antagonist injections resulted in an elevation in free feeding.

GLP-1R stimulation can induce nausea that may alter the action of GLP-1 on food intake (208, 223). However our results showed that microinjection of EX4 into the VTA or the NAc decreased chow intake at 24h time point without increasing the consumption of kaolin (Fig. 1.6). This result clearly indicates that the mesolimbic GLP-1R stimulation will reduce food intake without inducing malaise.

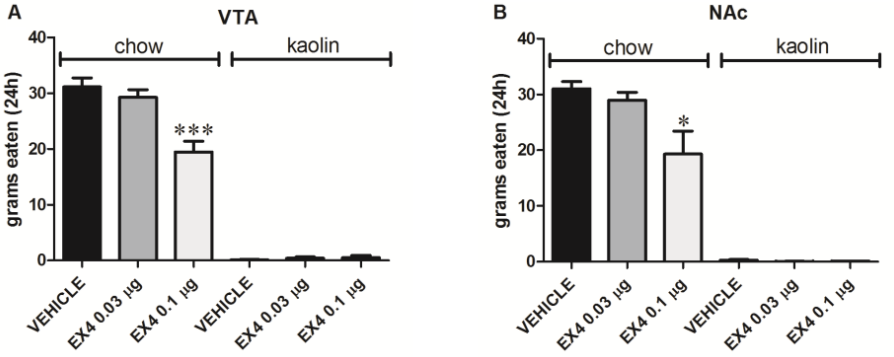


Figure 1.6. Direct microinjection of EX4 into the VTA (A) and the NAc (B) induced chow intake reduction is not accompanied by malaise response. N = 4-8. *p<0.05, ***p<0.0005. Data represent mean ± SEM.

GLP-1R are expressed in the CeA and GLP-1R blockade in this area has been previously shown to abolish the illness induced by lithium chloride (223). In addition, another study also has demonstrated that GLP-1R activation in the hypothalamus does not induce CTA (151). Furthermore current data suggest that the anorexic effect of GLP-1 in the VTA or the NAc is not caused by malaise. The lack of malaise response in the current data is in line with results obtained by (39, 155). These finding suggested that visceral illness is not associated with VTA and NAc GLP-1R induced suppression of food-oriented behavior.

In summary, we evaluated the impact of peripheral, central and intra-mesolimbic GLP-1R stimulation with EX4 on two well-known reward behaviors, CPP and operant conditioning.

- EX4 decreased reward behavior in the CPP test as rats treated with EX4 spent less time in chamber paired with chocolate during conditioning.
- Both peripheral and central administration of EX4 decreased motivated behavior in operant conditioning for sucrose.
- Intra-mesolimbic GLP-1R stimulation also decreased operant responding without inducing malaise.

These findings highlight the mesolimbic reward system as a novel site of action for GLP-1R-mediated food-oriented behavior.

Paper II

This paper follows up on the results obtained in Paper I, and aims to further investigate the neurocircuitry downstream of the VTA GLP-1 activation.

Here we reveal a new role of amygdala dopamine signaling in food-oriented behavior. We demonstrated that feeding increases dopamine turnover in the amygdala. In addition, D2 but not D1 receptor blockade increased food intake. We also showed the impact of central GLP-1R activation on amygdala dopamine turnover and its contribution to the anorexic effect of GLP-1.

An elevated level of dopamine metabolites, DOPAC and HVA, and dopamine turnover was seen after 30 minutes of chow intake in overnight food-restricted rats (Fig. 2.1). These data suggest that amygdala dopamine signaling is affected by feeding.

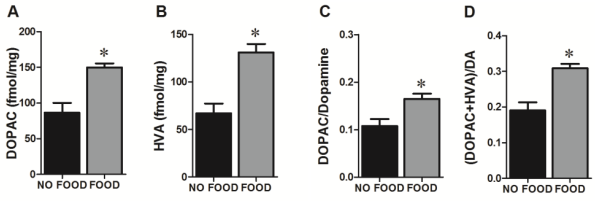


Figure 2.1. The impact of feeding on amygdala dopamine turnover. Tissue concentration of dopamine metabolites (A,B) and dopamine turnover (C,D) are measured. Values for HVA and DOPAC are expressed as fmol/mg brain tissue. N = 3-8. *p<0.05. Data represent mean ± SEM.

Intra-amygdala injection of D1 receptor agonist (SKF) and antagonist (SCH) did not have any impact either on chow consumption or on PR operant conditioning (Fig. 2.2).

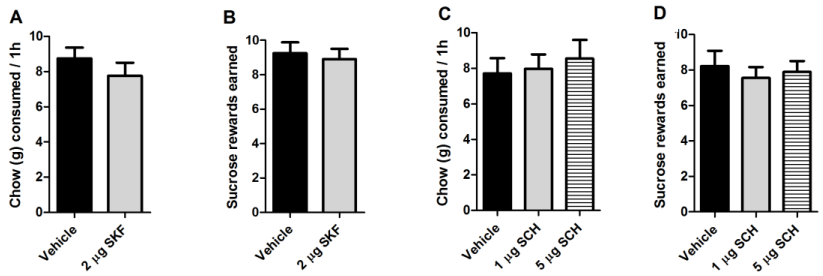


Figure 2.2. Effect of D1 receptor stimulation or blockade in the amygdala on food intake and food motivated behavior. Neither D1 receptor agonist nor antagonist altered chow intake (A, C) or operant responding for sucrose (B, D). N= 9-11. Data represent mean ± SEM.

In contrast to the results obtained with D1 receptor agonist, amygdala D2 receptor activation with QNP, reduced chow intake and lever pressing in the operant conditioning paradigm in both overnight food-restricted (Fig. 2.3. A-B) and satiated rats (Fig. 2.3.C).

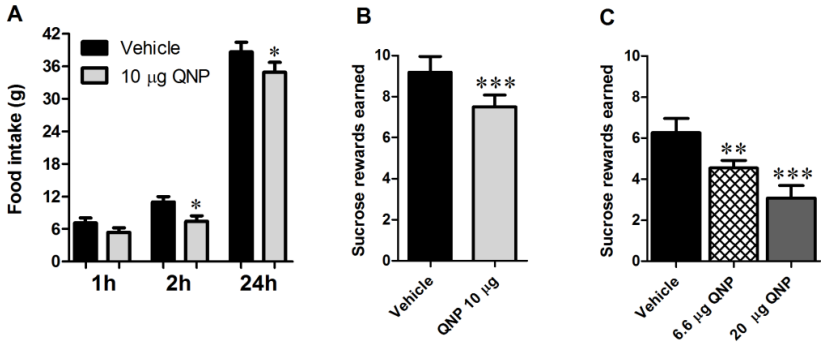


Figure 2.3. D2 receptor stimulation in the amygdala suppressed food intake and food motivated behavior. QNP decreased chow intake starting at 2h post injection (A). QNP also reduced the number of sucrose earned in operant conditioning in restricted (B) and in satiated (C) rats.). N= 9-11. *p<0.05. **p<0.005, ***p<0.0005 comparing vehicle to each QNP dose. Data represent mean ± SEM.

The amygdala D2/D3 blockade with ETC significantly increased chow intake at 1 h and 2 h (Fig. 2.4.A) but decreased the number of sucrose rewards earned under a PR reinforcement schedule in satiated rats (Fig. 2.4.B). The same

treatment in overnight food-restricted rats did not alter their operant performance but their food intake was elevated at 1 h after operant testing (Fig. 2.4. C-D).

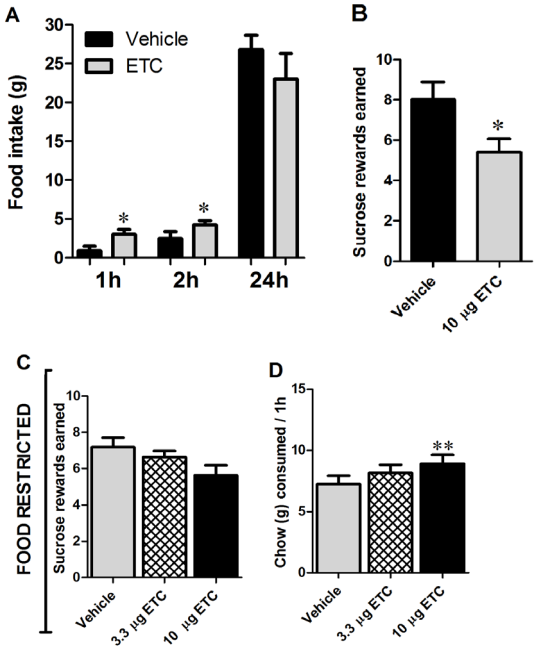


Figure 2.4. Effect of intra-amygdala microinjection of D2 antagonist, ETC on food intake and food motivated behavior. ETC Suppressed chow intake at 1 h and 2 h post injection (B). ETC decreased operant responding for sucrose in fed (A) but not food restricted rats (C). However, ETC increased food intake in food- restricted rats (D). *$p < 0.05$, **$p < 0.005$ comparing vehicle to each ETC dose. N=6-11. Data represent mean ±SEM.

The level of dopamine metabolites (DOPAC and HVA) and dopamine turnover was elevated after central injection of EX4 (0.3 µg) (Fig. 2.5). These data indicate that central GLP-1R stimulation will impact dopamine signaling in the amygdala.

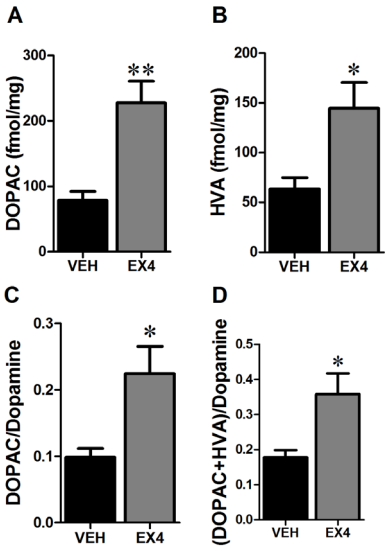


Figure 2.5. Impact of central GLP-1R stimulation on amygdala dopamine turnover. The concentration of DOPAC and HVA (A-B) and dopamine turnover (C-D) were measured after central EX4 administration in the amygdala. Values for HVA and DOPAC are expressed as fmol/mg brain tissue. N = 6-8. *p<0.05. Data represent mean ± SEM.

To investigate whether the anorexic effect of EX4 is mediated through the amygdala dopamine signaling, pretreatment with a selective D2/D3 antagonist, ETC, prior to central EX4 injection on food-oriented behavior was tested. Central EX4 administration reduced 1 h chow intake and pretreatment with ETC failed to block this reduction (Fig. 2.6. A). However, ETC attenuated the 24 h chow intake reduction induced by EX4 (Fig. 2.6. B), which indicated an interaction between ETC and EX4. EX4 decreased the number of sucrose pellets earned under PR operant conditioning. However, blockade of D2/D3 receptor by ETC did not attenuate EX4 induced operant behavior reduction (Fig. 2.6. C).

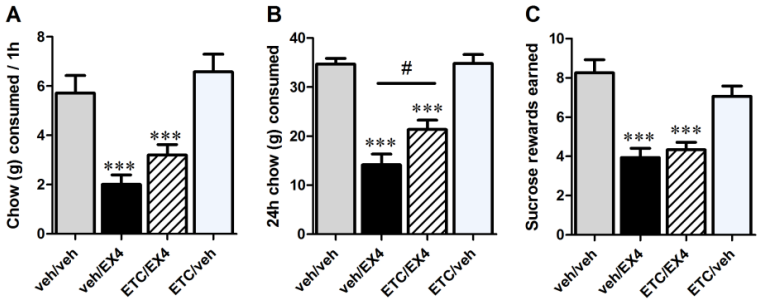


Figure 2.6. The impact of pretreatment with D2 antagonist, ETC, on central EX4-induced reduction on food intake and food reward. Pretreatment with ETC attenuated the suppressive effect of EX4 on food intake through a 24 h chow consumption (A-B) without altering the operant performance (C). ***$p < 0.0005$ compared to vehicle, # EX4 compared to ETC/EX4 group using the Tukey test. N=15. Data represents mean ± SEM.

We demonstrated that thirty minute food intake after a mild food restriction increased the level of dopamine turnover in the amygdala. These findings are in line with previous studies showing that amygdala dopamine level was increased by feeding (105, 224). Since central dopamine is involved in motor activity and feeding requires motor effort to approach, chew and swallow the food, it can be difficult to interpret the results in these feeding experiments without knowing whether motor behavior was intact. However, both intra-gastric infusions of nutrients and peripheral administration of glucose increased the amygdala dopamine level, which supports the hypothesis that the dopamine elevation is dependent on an increase in nutrients and not motor activity (105, 224).

D1 and D2 receptors are expressed in the amygdala (103). Activation of amygdala D2 receptors resulted in a reduction of chow intake in both food-restricted and satiated rats, suggesting that the activation of amygdala D2 receptors is sufficient to attenuate food intake. The endogenous role of amygdala dopamine in feeding is highlighted after intra-amygdala injection of D2 antagonist which increased the amount of food eaten. Amygdala D2 receptor stimulation by QNP decreased the food reward behavior. This finding is consistent with the one obtained by Simon et al. (101) which suggested that amygdala dopamine lesion enhances amphetamine self-administration. Previous studies have shown that systemic administration of amphetamine will reduce both operant responding and chow intake (225, 226). In these studies D1 and D2 antagonist suppressed operant behavior but increased chow intake; that is in line with our findings. In the current study blockade of amygdala D2 receptor with ETC resulted in a decrease in food reward in satiated rats, which is

surprising, but this effect has also been seen in previous studies (225, 227). Intra-amygdala blockade of D3 attenuated Pavlovian conditioning whereas its stimulation enhanced Pavlovian conditioning (228, 229). Thus, it is important to note that the reduction in operant behavior in our study can be due to blockade of D3 receptors since ETC can bind to both D2 and D3 receptors.

Considering the abundant expression of GLP-1R in the amygdala, we showed that central EX4 administration elevated the level of dopamine turnover in this nucleus. This elevation of dopamine is necessary for the anorexic effect of central GLP-1R stimulation. However, D2 receptor antagonist, ETC, failed to block the total effect of EX4 induced reduction in food intake and operant responding. As mentioned earlier GLP-1R are expressed in other brain nuclei such as the VTA, the NTS, and the hypothalamus. The current result is only focusing on the amygdala dopamine signaling and the lack of effect of ETC on EX4 induced reduction in operant response implicates that other brain nuclei might be involved in central action of GLP-1R activation on food reward behavior. In the current study EX4 was applied in the LV which may reach other brain nuclei such as NTS and VTA. Activation of VTA GLP-1R resulted in a reduction in food intake (39) and food reward behavior as demonstrated in paper I. The hindbrain and central GLP-1 neurons are activated by both food consumption and viscerosensory stress (230-232). This activation can induce illness and CTA since pharmacological blockade of CNS GLP-1R attenuated Pica response and CTA induced by lithium chloride (207). Activation of NTS and amygdala GLP-1R induces nausea and CTA (208, 223) whereas stimulation of VTA GLP-1R does not induce illness as described in paper I and by Alhadeff et al. (39). However recent data demonstrate that NTS GLP-1R stimulation with EX4 at a dose that reduces food reward behavior, does not induce the pica response (42). The role of amygdala D2 receptors in illness has not been studied. Nevertheless, D3 receptors in the AP may contribute to nausea (233). Collectively our data implicate that VTA GLP-1R and amygdala D2 receptors reduce food reward without inducing illness.

In conclusion, we demonstrated the effect of GLP-1 on the amygdala dopamine system on food-oriented behavior;

- Food intake increased the level of amygdala dopamine.
- Stimulation of amygdala D2, but not D1, receptor reduced intake of chow and PR operant behavior for sucrose reward.

- Blockade of D2 receptors in the amygdala increased chow intake. Central GLP-1R stimulation with the long lasting agonist EX4 elevated dopamine turnover in the amygdala and this elevation contributed to the anorexic effect of GLP-1.

Taken together we revealed a novel role of the amygdala D2 receptor on food intake.

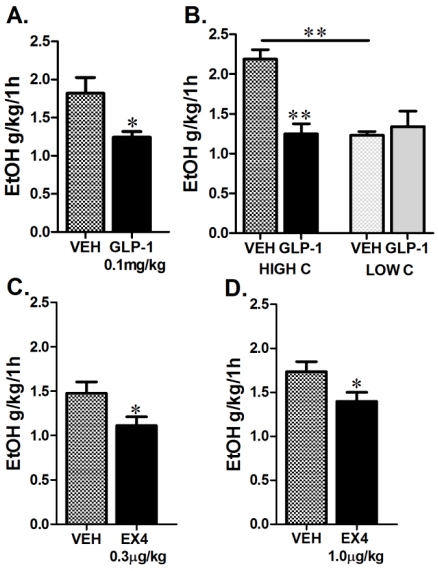
Paper III

The previous two papers focused on the reward derived from food, the so-called natural reward. However the neural substrates underlying natural rewards are also known to control reward derived from drugs of abuse or alcohol. Thus, in Paper III the focus is shifted to the role of GLP-1 in regulation of reward behavior derived from alcohol.

In this paper we demonstrated the effect of GLP-1 and its analogue, EX4, on alcohol intake and reward when injected either peripherally or centrally. We also highlight the mesolimbic VTA as a novel target for GLP-1 to reduce alcohol consumption.

Peripheral injection of GLP-1 reduced alcohol intake by 30% compared to vehicle in a voluntary alcohol drinking paradigm (Fig. 3.1A). The rats from this study were later analyzed as high and low drinking groups based on their vehicle baseline drinking. The interesting outcome of this division was an interaction between the GLP-1 effect and the baseline drinking. GLP-1 decreased alcohol intake only in the high drinking group (Fig. 3.1B). However, this division was not essential to discover the effect of GLP-1 on drinking, since GLP-1 reduced alcohol intake when all rats were included as shown in fig. 3.1A. GLP-1 did not affect either water or chow intake (data not shown).

Alcohol intake was reduced by I.P. EX4 (0.3 and 1.0 μg/kg) (Fig. 3.1 C, D). The lower dose of EX4 did not alter water or food intake, which supported the selective effect toward only alcohol not general liquid intake. However, the higher dose decreased water intake without altering chow intake.



Figur 3.1. Peripheral GLP-1 or EX4 reduced alcohol consumption in an intermittent-access 20% alcohol drinking paradigm. A) Peripheral GLP-1 injected decreased alcohol intake compare to vehicle group (N = 12 per treatment group). B) A significant alcohol intake reduction was seen in high alcohol consumption rats but not in low consumption. C, D) Peripheral injection of EX4 at a dose of either 0.3 or 1.0 μg/kg attenuated alcohol intake N = 13-25. *p<0.05, **p<0.0005. Data represent mean ± SEM.

In NMRI mice, daily alcohol injection induced a preference for the chamber it was paired with (see methods section for CPP) during the conditioning days. On the testing day, mice injected with saline spent significantly more time in the chamber previously paired with alcohol. In contrast GLP-1 treated mice spent the same amount of time in both chambers (saline vs. alcohol) (Fig. 3.2.A). Thus, GLP-1 reduced %CPP on the testing day (Fig. 3.2.B). This result indicates that peripheral GLP-1R activation reduces the reward derived from alcohol.

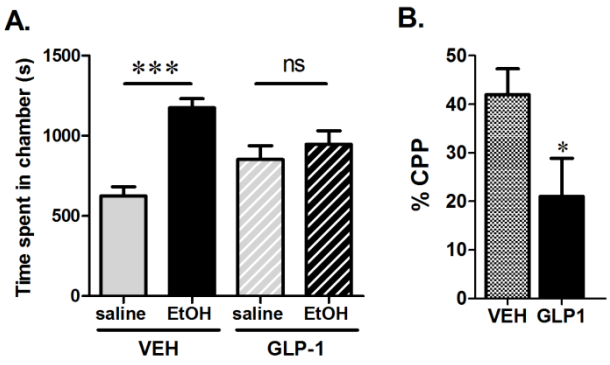


Figure 3.2. Peripheral GLP-1 administration decreases alcohol reward. A) On testing day, mice treated with saline showed a preference for the chamber paired with alcohol during conditioning days. But mice treated with 0.02 mg GLP-1 spent equal time in both chambers. B) Alcohol induced a preference in saline (N = 48) but not in GLP-1 (N = 31) treated mice. %CPP was calculated as follow ((test-pretest)/(total time pretest)) to indicate a preference above a natural response. *p<0.05, ***p<0.0005. Data represent mean ± SEM.

To assess the role of endogenous GLP-1 on alcohol intake, rats were injected peripherally with the GLP-1R antagonist, EX9. The peripheral EX9 injection led to a trend to increase alcohol intake at 1h (Fig. 3.3 A) and increased alcohol intake at 24h (Fig. 3.3 B). EX9 did not alter either food or water intake in the alcohol-consuming Wistar rats under current experimental conditions.

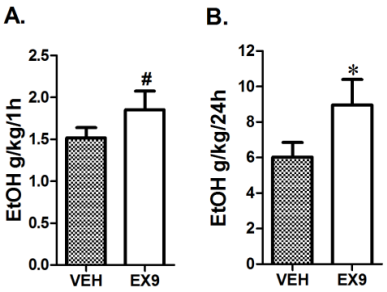


Figure 3.3. Peripheral GLP-1R blockade increased alcohol consumption in free alcohol drinking paradigm. A) Peripheral administration of 0.1 mg/kg EX9 displayed a tendency to increase 20 % alcohol intake at 1h. B) Alcohol intake was significantly increased by EX9 at 24h. N = 12-13. #p<0.1, *p<0.05. Data represent mean ± SEM.

To uncover the neural substrate underlying these effects, we injected GLP-1 and EX4 into the VTA. Intra-VTA GLP-1 injection attenuated alcohol intake only at 16h, but not at 1h (Fig. 3.4 A, B). The VTA GLP-1 injection did not alter chow or water intake. Direct EX4 injection into the VTA resulted in a decrease in alcohol intake during 1h and 16h drinking session (Fig. 3.4 C, D) but it also reduced water intake. Intra-VTA EX4 did not affect 1h chow intake but suppressed the 16h food intake.

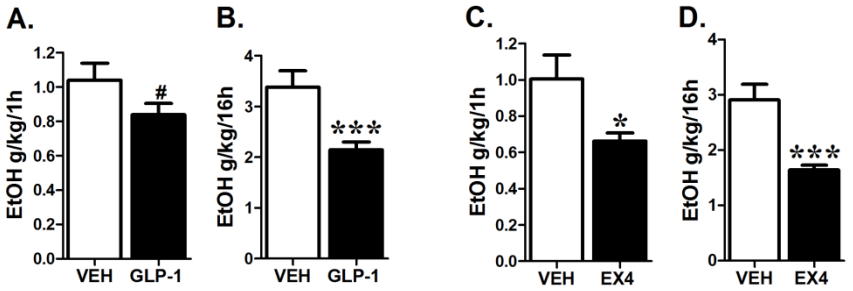


Figure. 3.4. Identification of the mesolimbic VTA, as a site of action of GLP-1´s effect on alcohol consumption. Intra-VTA microinjection of GLP-1 (vehicle N= 11; GLP-1 1 mg, N=7, A -B) and EX4 (vehicle N=9; EX4 0.1 mg N=9, C-D) suppressed 20% ethanol intake. $^{\#}p<0.1$, $^{*}p<0.05$. $^{***}p<0.0005$. Data represent mean ± SEM.

Peripheral administration of GLP-1 decreased ethanol intake and reward. The GLP-1 long lasting analogue EX4 also suppressed alcohol intake when injected peripherally or centrally. These results demonstrate that GLP-1R stimulation is sufficient to attenuate alcohol-oriented behavior. Our results also suggest that GLP-1R activation in the mesolimbic VTA reduces alcohol intake but does not rule out the role of other GLP-1R-expressing brain regions that also receive dopaminergic projections from the VTA, such as NAc. These results are in line with another study (234) that demonstrated a decrease in accumbal dopamine release in mice after peripheral injection of EX4 using microdialysis. In this study peripheral injection of EX4 attenuated alcohol induced CPP in mice and alcohol self-administration in an operant test in rats (234) further supporting our results. Importantly, recent evidence demonstrates that the level of TH, a key enzyme in dopamine synthesis, is increased after VTA GLP-1R stimulation (41). Peripheral blockade of endogenous GLP-1 elevated alcohol consumption which implicates the role of endogenous GLP-1 in regulation of alcohol intake. Alcohol can be a source of calories and fluids along with its direct rewarding effect. Thus, it should be noted that GLP-1 might potentially reduce calorie

intake rather than alcohol consumption. However, the collected data here clearly show that both central and peripheral GLP-1R stimulation reduced alcohol consumption without affecting water or chow intake. Furthermore, EX9 did not alter either chow or water intake, while it increased alcohol intake. This dissociation was only seen in the lower dose of peripherally administrated EX4 whereas the higher dose and intra-VTA injections attenuated water intake.

In conclusion, we assessed the effect of GLP-1R stimulation on alcohol reward and intake.

- Peripheral GLP-1R stimulation attenuated alcohol reward in the CPP test.
- Peripheral injection of GLP-1 or its analogue, EX4, decreased alcohol intake in a free drinking paradigm.
- The impact of endogenously released GLP-1, evaluated by application of the GLP-1 antagonist, EX9, was suggested by increased alcohol intake after the antagonist treatment.
- Direct VTA GLP-1R stimulation reduced alcohol intake.

Taken together, these results reveal a new role of GLP-1 system in modulating alcohol intake and reward.

Paper IV

While the first three papers focused on the impact of GLP-1 on reward behavior driven by GLP-1R in brain nuclei previously unexplored with respect to GLP-1, Paper IV focused on the hypothalamus and basic feeding behavior – so a brain nucleus well-known to participate in physiological responses to GLP-1. The novelty of this paper lies in the choice of mediators of GLP-1 – molecules associated with the immune response.

In this paper we showed an interaction between GLP-1, IL-6 and IL-1β in regulation of food intake and body weight in the brain by using both pharmacological and genetic models of blockade/deficit of IL-6 and IL-1. We also showed that central GLP-1R stimulation increases the level of IL-6 and IL-1β in both hypothalamus and hindbrain.

Central GLP-1R stimulation with EX4 elevated the level of IL-6 and IL-1β mRNA in the hypothalamus, and only IL-6 in the hindbrain without affecting other cytokines in rats fed *ad libitum* IL-6 mRNA elevation in the hypothalamus was 11 fold higher compared to vehicle while the IL-1β mRNA was three fold higher compared to vehicle treated rats (Fig. 4.1. A, C). The food restricted group showed a larger variability in gene expression. Food restriction alone did not have any significant effect on cytokine gene expression measured in this study. However, the EX4 effect was dampened in the food restricted rats (Fig. 4.1. B, D). The results are normalized to β-actin.

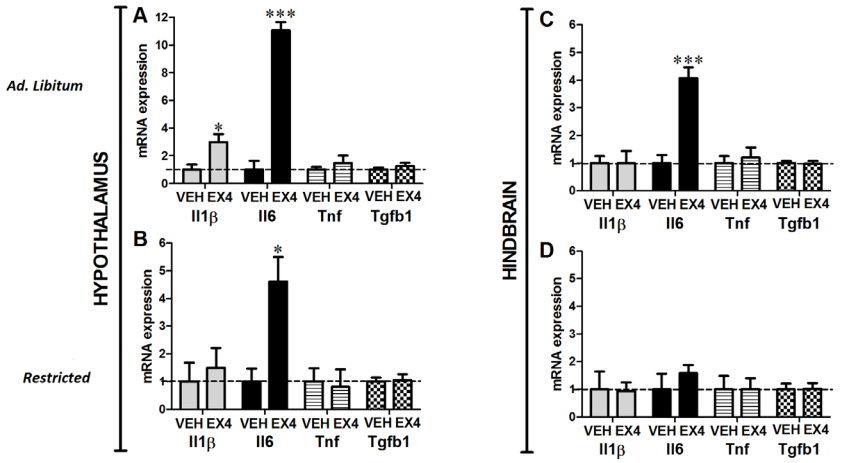


Figure 4.1. ICV injection of EX4 increased significantly the level of IL-6 in both hypothalamus and the hindbrain in ad.lib (A, C) and in the food restricted rats (B, D). N=9-11 per treatment. **p*<0.05, ****p*<0.0005. Data represent mean ± SEM.

In line with the elevation of IL-6 and IL-1β, central EX4 administration also increased the level of interleukin-associated intracellular signals, namely phosphorylation of signal transducer and activator of transcription-3 (pSTAT3) and suppressor of SOCS1 and SOCS2 (221) in the hypothalamus. Only pSTAT3 was detected in the DVC (Fig. 4.2. A-D).

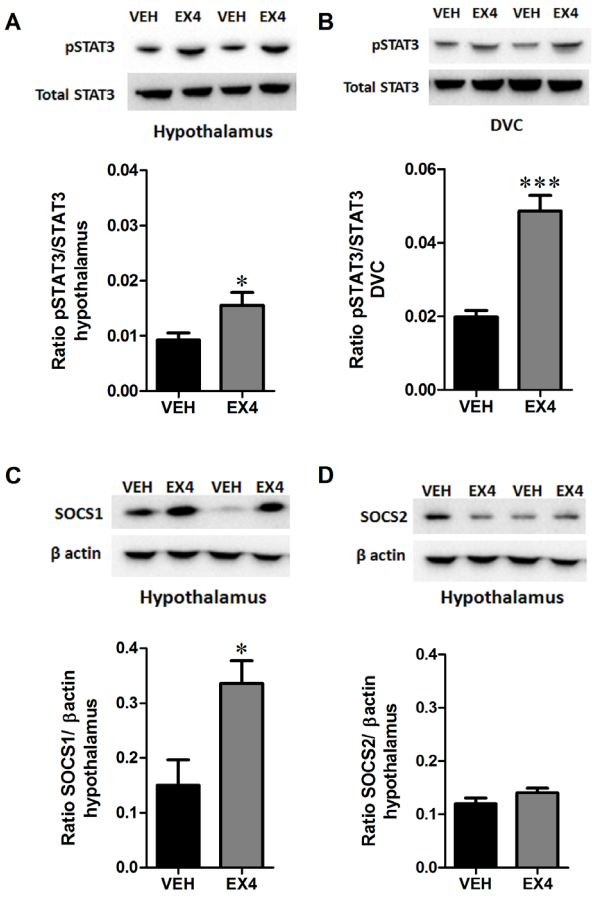


Figure 4.2. Central GLP-1R stimulation with EX4 increased the level of interleukin-associated intracellular signals in fed rats. Central EX4 injection induced an elevation in phosphorylation of STAT-3 in the hypothalamus (A) and the DVC (B). EX4 increased also the protein level of hypothalamic SOCS1 (C) but not SOCS2 (D). N = 3/treatment. *p<0.05, ***p<0.0005. Data represent mean ± SEM.

We also found that mRNA expression of POMC was elevated in both the hypothalamus and the hindbrain (Fig. 4.3 A-B). In contrast, EX4 did not alter the level of other anorexic/orexigenic peptides. This result is in line with data previously obtained by Koole et al. (235) indicating that melanocortins are a downstream signal of IL-1β in the hypothalamus. However, it is also possible that hypothalamic GLP-1 induced IL-1β elevation can contribute to increase in POMC expression.

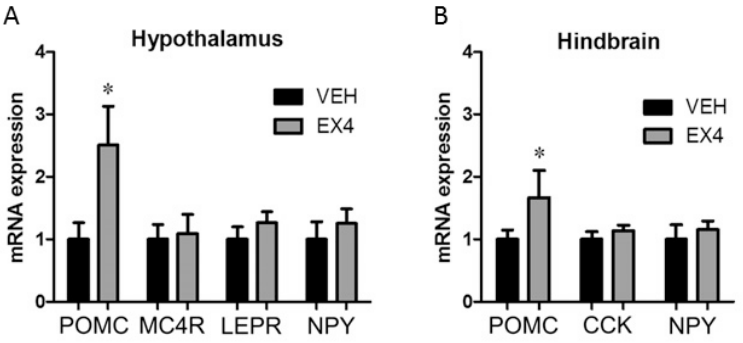


Figure 4.3. Central EX4 administration elevated the level of POMC in both hypothalamus and hindbrain without altering the expression of other neuropeptides. N = 3/treatment. *p<0.05, ***p<0.0005. Data represent mean ± SEM.

To determine the physiological relevance of this interaction, we used both genetic and pharmacological deficit/blockade models of IL-6 and IL-1 signaling. Our results indicate that pretreatment with IL-6 or IL-1R antagonist did not alter EX4-induced food reduction at 4 h post injection (Fig. 4.4 A, D). In contrast it significantly attenuated EX4-induced food reduction at 22 h post injection (Fig. 4.4 B, E). EX4-induced body weight loss was partially abolished by IL-6 blockade whereas IL-1R blockade completely blocked body weight loss induced by EX4 (Fig.4.4. C, F).

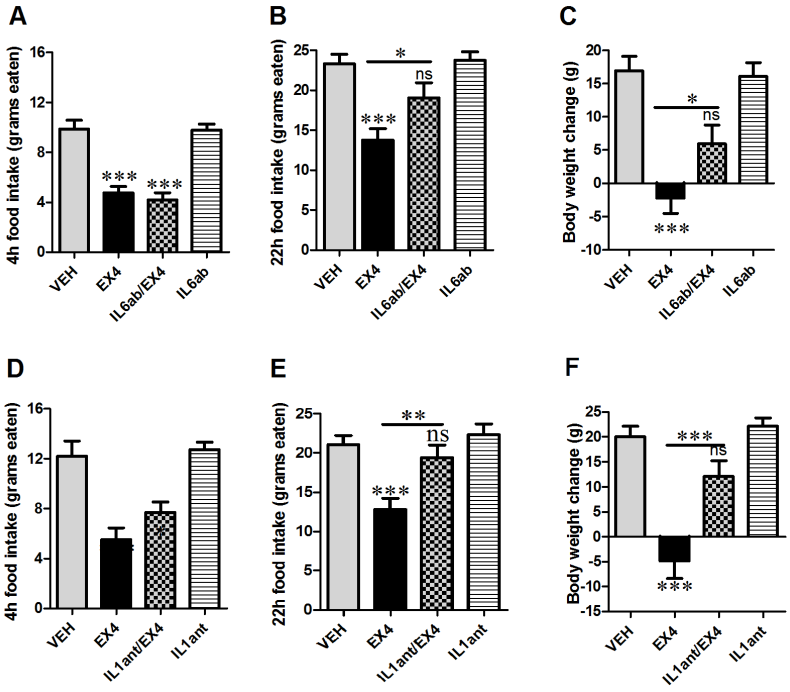


Figure 4.4. Central (ICV) co-administration of IL-6 and IL-1R blockade attenuated food intake and body weight loss induced by EX4. IL-6ab and IL-1Ra did not alter EX4-induced chow intake reduction after 4 h post injection (A, D). However, they blocked the anorexic effect of EX4 at 22 h time point (B, E). IL-6ab and IL-1Ra dampened EX4-induced body weight loss (C, F). N = 12-16 /treatment group. *p<0.05. **p<0.005, ***p<0.0005. Data represent mean ± SEM.

The lack of short-term effect of either IL-6 or IL-1R blockade on EX4 induced anorexia might be due to a potential redundancy between IL6 and IL-1. To evaluate this hypothesis, rats were pretreated simultaneously with IL-6 and IL-1R blockade. This combination blockade of IL-6 and IL-1R attenuated the anorexic effect of EX4 and EX4 induced body weight loss (Fig. 4.5). This synergistic effect on EX4 induced chow intake reduction was already seen at 1 h compared to IL-6 or IL-1R blockade alone. In line with our results, mice lacking both IL-6 and IL-1 develop obesity earlier than mice lacking either interleukins alone (180).

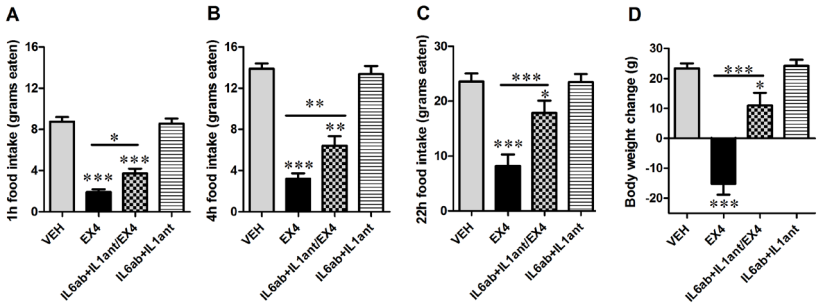


Figure 4.5. A simultaneous IL-6 and IL-1R blockade attenuated EX4 induced intake and body weight reduction. N = 11/treatment. *p<0.05. **p<0.005, ***p<0.0005. Data represent mean ± SEM.

During infection an elevated level of cytokines is associated with a high body temperature and locomotor depression. Some studies also show that GLP-1R stimulation with EX4 will induce hypoactivity. It has also been shown that GLP-1 can affect body temperature (160). To confirm that our behavioral results obtained here are not due to behavioral suppression induced by EX4, we measured both core body temperature and activity. The results suggested no significant alteration in activity and surprisingly in one experiment, EX4 increased activity (Fig. 4.6 A-C). Central injection of EX4 whether alone or in combination with IL-6 or IL-1R blockade, induced hypothermia. Single injection of IL-6 or IL-1R blockade, did not induce reductions in body temperature (Fig. 4.6 C-D). This effect persisted up to 4 h post injection. However, the simultaneous IL-6 and IL-1R blockade partly attenuated the hypothermic effect induced by EX4 (Fig. 4.6 F).

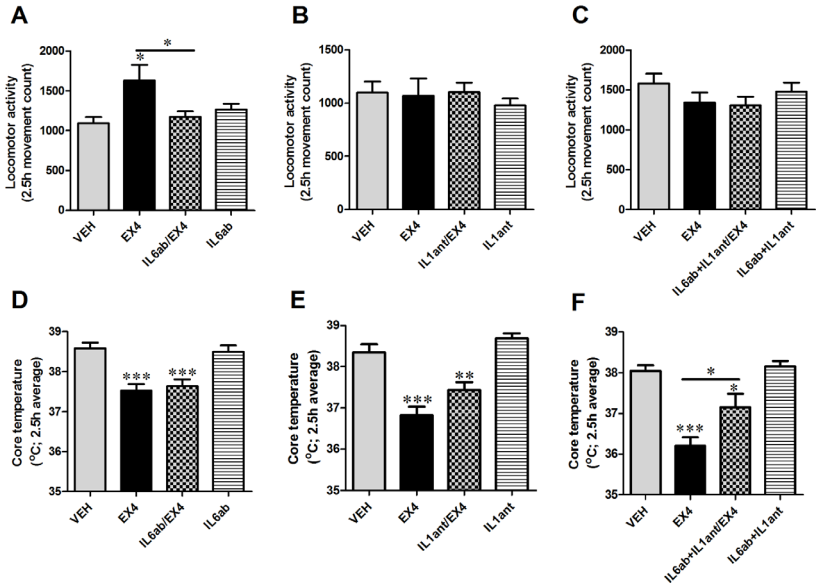


Figure 4.6. Activity and core temperature are measured after central GLP-1R stimulation with EX4 alone or in combination with IL-6 or IL-1 blockade. Motor activity was not altered by single or combination administration of either EX4 or IL-6 or IL-1 (A-C). Central EX4 injection reduced core temperature and IL-6 or IL-1R blockade alone did not attenuated the hypothermic effect of EX4 (D-E). Meanwhile a combination of IL-6 and IL-1R antagonist abolished EX4 induced hypothermia (F).

In addition to pharmacological blockade, EX4 was peripherally injected to genetically IL-6Rα- or IL-1-deficient mice. Peripheral administration of EX4 to control mice reduced chow intake at 4 h and 22 h post injection compared with vehicle treated mice. A reduction in body weight was also observed in these mice (Fig. 4.7A, C). But IL-6Rα knockdown and IL-1R knockout mice reduced their chow intake only at 6 h post injection whereas no significant effect was seen at 22 h. The body weight was not altered in either of the mouse groups (Fig. 4.7B, D).

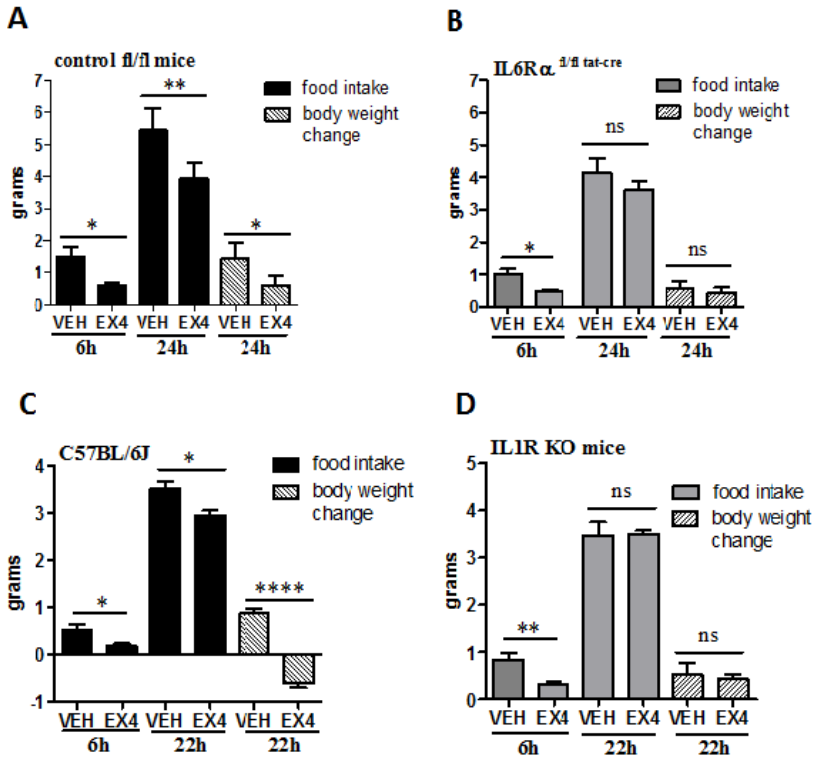


Figure 4.7. Anorexic effect of EX4 is attenuated in IL-6Rα and IL-1R deficit mice. Peripheral administration of EX4 reduced chow intake and body weight in control mice (A, C) but it did not alter suppress feeding and body weight at 22 h point in IL-6Rα and IL-R deficit mice (B, D). N = 8 (A, B), N = 5 (C), N = 6 (D). *p<0.05. **p<0.005, ***p<0.0005. Data represent mean ± SEM.

The results obtained from the behavioral studies indicate that central IL-6 and IL-1 signaling is not required for the short-latency EX4 mediated anorexia but are necessary mediators for the longer-term anorexic effect of EX4.

Studies have indicated that IL-6 and IL-1β play a critical role in the regulation of energy balance mediated by the fat producing hormone leptin (170, 236, 237). These studies fit well with our observation here showing that the anorexic effect of GLP-1 is mediated by IL-6 and IL-1β. In fact, past literature has indicated that GLP-1 is a mediator of leptin induced food intake reduction and body weight loss (238, 239).

Both IL-6 and IL-1 have been suggested to be involved in the regulation of food intake and body weight during health (170, 177, 178) and infection induced anorexia (240).

The hypothermic effect of EX4 observed here, is in line with previous studies (153, 160, 161). Body temperature is often increased due to an increased level of IL-6 and IL-1. However, in one previous study IL-1β induced hypothermia in some circumstances which makes it a possible mediator of GLP-1R induced hypothermia (241) as observed here after EX4 administration. The specificity of the transcriptional modulation induced by central GLP-1R stimulation is highlighted by the fact that central EX4 administration elevated selectively IL-6 and IL-1β without impacting other cytokines and inflammatory-associated molecules such as TNFα. Moreover, locomotor depression, a common effect of systemic inflammation, is not observed here after central administration of EX4 or interleukin blockade. These data support the conclusion that both, cytokine profiles and behavior induced by GLP-1R stimulation, differ from those induced by general inflammation. Moreover, GLP-1R stimulation has been demonstrated to diminish the CNS inflammatory response to LPS administration or irradiation (183, 242, 243). This finding strengthened the hypothesis that the anorexic effect of GLP-1R stimulation could be mediated via IL-6 and IL-1 at the level of the CNS.

In summary results obtained here suggest that:

- Central stimulation of GLP-1R increased the levels of IL-6 and IL-1 in hypothalamus and hindbrain, two key brain nuclei involved in energy balance regulation, without altering other cytokines.
- Central stimulation of GLP-1R also increased the level of POMC and signals downstream of interleukins in these nuclei.
- Pharmacological blockade of CNS IL-6 or IL-1R attenuated anorexia and body weight reduction induced by central GLP-1R activation.
- The same effect was seen in the mice with IL-1R knock-out or IL-6Rα knock-down.

Taken together our results provide evidence that the anorexic and weight loss effect of GLP-1 are mediated via IL-6 and IL-1 at the level of the brain.

Concluding remarks

Obesity primarily driven by excessive intake of palatable food is a major worldwide health concern. Palatable, energy dense, food can exert a reinforcing effect that is mediated by the brain reward circuitry, the mesolimbic system. Thus, reducing the rewarding value of food may provide a new therapeutic direction for the treatment of obesity. Feeding behavior is controlled by a complex and distributed neural pathway involving a variety of neuropeptides and neurotransmitters. Dysregulation of the reward pathway can alter food reward and lead to obesity.

The focus of this thesis was to investigate the role of GLP-1 on food and alcohol reward behavior and to identify the neurocircuitry and neurochemical mediators involved. Furthermore a surprising link, relevant for anorexic and weight loss effect of GLP-1, between GLP-1 and cytokines, was discovered.

We have demonstrated that GLP-1 and its long lasting analogue, EX4, can modulate the rewarding value of palatable food (e.g. sugar) and drugs of abuse (e.g. alcohol) by specifically targeting the reward circuitry nuclei, VTA and NAc. Central GLP-1R stimulation not only reduced food and alcohol intake but also reduced the rewarding value of both of these substances. Motivation to obtain palatable food was also suppressed by central GLP-1R activation. In addition, an alteration in dopamine signaling at the level of the amygdala contributed to the anorexic effect of GLP-1.

In addition to the effect of GLP-1 on hedonic behavior, central GLP-1R activation also increased the hypothalamic and hindbrain levels of two inflammatory cytokines, IL-6 and IL-1, without inducing fever. Both IL-6 and IL-1 were revealed to be downstream mediators of GLP-1 induced suppression of food intake and body weight. Whether the elevation of IL-6 and IL-1β might contribute to the suppression effect of GLP-1 on reward behavior remains to be investigated.

In summary, the data in this thesis support the hypothesis that central GLP-1 can regulate metabolism and reward behavior through its action on several distributed brain regions. However, we have to keep in mind that most of the evidence presented here resulted from an acute GLP-1 effect on feeding behavior. Long-term studies are therefore now needed to confirm these findings

in a chronic setting that would more closely reflect the clinical application of GLP-1 analogues.

Considering that GLP-1 analogues are already approved for treatment of T2DM and obesity, these findings are of considerable clinical significance. Yet, further clinical studies are needed to unravel the potential pleiotropic effects of these agents in a human population.

Populärvetenskaplig sammanfattning

De senaste decennierna har fetma och övervikt blivit ett stort globalt problem och dessa åkommor är idag viktiga riskfaktorer för utveckling av bl.a. hjärt- och kärlsjukdomar samt typ 2 diabetes. Orsaken till fetmaepidemin är multifaktoriell men givetvis är grundorsaken att intaget av energi via födan är större än kroppens energiåtgång. En viktig faktor är att viss kaloririk mat kan påverka hjärnans belöningscentrum på samma sätt som beroendeframkallande ämnen, såsom alkohol och droger. Hjärnans belöningssystem är en väldefinierad struktur som kan ge en välbehagskänsla (eufori) när det aktiveras av naturliga (mat, sex) eller icke-naturliga (alkohol, droger) signaler. Euforin betingas av frisättning av dopamin i hjärnans belöningscentrum. Belöningssystemet innefattar två viktiga områden; ventral tegmental area (VTA) och nucleus accumbens (NAc) där VTA innehåller dopaminproducerande celler som projicerar till bl.a. NAc och amygdala. Då överkonsumtion till viss del anses bero på en rubbning i detta system finns det idag ett stort behov av nya effektiva mediciner för att bekämpa övervikt.

På senare år har man börjat behandla vissa typ 2 diabetiker med glukagon-like peptide-1 (GLP-1)-baserade läkemedel. Det första läkemedlet i denna grupp var exendin4 (EX4), en syntetisk och långverkande version av kroppens eget GLP-1. EX4 och andra GLP-1-analoger förbättrar sjukdomstillståndet genom att sänka blodets glukosnivåer, det leder också till måttlig viktnedgång. Efter varje måltid ökar nivåerna av GLP-1 i blodet vilket leder till ökad insulinproduktion samt en aptitdämpande effekt. GLP-1 produceras både i tarmen och i hjärnan och GLP-1 receptorer (GLP-1R) är distribuerade i olika hjärnområden t.ex. hypotalamus, VTA, NAc och amygdala. Flera studier har visat att aktivering av hjärnans GLP-1R dämpar aptit och minskar kroppsvikt. Nyligen har en GLP-1-analog (liraglutide) blivit godkänd för behandling av fetma i USA och Europa. Det är därför viktigt att undersöka mekanismerna bakom GLP-1 effekter samt utreda potentiella biverkningar som kan uppstå efter behandling med dess preparat.

Genom att använda två väletablerade beteendemodeller, operant conditioning och conditioned place preference, studerade vi effekterna av GLP-1 på belöningssystemet. Med operant conditioning undersöker man hur motiverade djur är för att få en belöning, i detta fall lär de sig att trycka på ett spak för att erhålla en belöning i form av en 45mg sockertablett. Andelen arbete som råttan

måste uträtta för att erhålla varje pellet ökar exponentiellt och man kan på så sätt undersöka hur motiverade de är för att få sockertabletten. Conditioned place preference (CPP) består av två sammankopplade kammare med olika visuella egenskaper. Testet omfattar tre faser; habituation, conditioning och preference test. Under conditioning-dagarna har djuret endast tillgång till ena kammaren, den minst föredragna kammaren paras ihop med ett belönande ämne såsom choklad eller alkohol, den andra kammaren paras ihop med ett mindre givande ämne liksom normal mat eller saltlösning. Under testdagen har djuret tillgång till båda kammarna efter injektion av GLP-1, EX4 eller saltlösning.

Vi fann att aktivering av GLP-1R minskade den belönande effekten av mat och alkohol i både operant conditioning och CPP. Aktivering av VTA GLP-1R var också tillräcklig för att minska suget efter mat och alkohol. Blockering av GLP-1R kunde minska effekten av EX4 i operant conditioning. Dessutom ökade råttornas intag av alkohol efter GLP-1R blockering.

Amygdala har förknippats med känslomässiga processer, inlärning och minne men endast ett fåtal studier har focus på amygdalas roll i födointag. Studier har visats att mygdala innehåller GLP-1R. I denna avhandling undersökte vi om GLP-1 interagerar med dopamin i amygdala, och vi visade därmed att GLP-1R-stimulering ökar dopaminnivåerna i detta område.

Ett antal studier har visat en koppling mellan GLP-1 och inflammatoriska faktorer, nämligen interleukin-6 (IL-6) och interleukin-1β (IL-1β). Det har även visats att transgena möss som saknar IL-6 eller IL-1β, eller bägge, utvecklar fetma. Nyligen har en studie visat att höga blodnivåer av IL-6 kan leda till en ökning av GLP-1-produktion och frisättning. För att undersöka om det finns en koppling mellan GLP-1, IL-6 och IL-1β i CNS använde vi både farmakologiska och icke-farmakologiska (transgena möss) modeller. Våra resultat indikerar den anorektiska effekten som uppstår efter administration av GLP-1 medieras av IL-6 och IL-1β.

Våra fynd visar en potent effekt av GLP-1 på mat- och alkohol-motiverat beteende och indikerar också vilka neuroanatomiska strukturer och neurokemiska substanser som är relevanta för den anorektiska effekten och viktminskningen som uppstår efter behandling med GLP-1. Vidare hittade vi även en koppling mellan GLP-1 och cytokiner i CNS.

Sammanfattningsvis kan de aktuella fynden ha stor klinisk betydelse eftersom GLP-1-baserad terapi har blivit en ny, potentiell behandling för fetma och våra data kan lämna ett viktigt bidrag till förståelsen av dessa mekanismer.

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